UNIVERSIDAD AUTÓNOMA DE MADRID

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TESIS DOCTORAL

Vadir López Salmerón

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Vadir López Salmerón

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Vº Bº del Tutor: Vº Bº del Director:

Dr. Rafael Pérez Mellado **Dr. Salomé Prat**

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Durante el trabajo realizado en esta tesis me he encontrado con personas maravillosas que han contribuido no solo profesionalmente sino también personalmente. Aún recuerdo aquel día que entre por primera vez al 314. Sin duda, este trabajo ha sido fruto de muchas horas invertidas y compartidas con tres personas; Cristina, JeanMi y Salomé.

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A Amaia:

Lao-Tsè decía: “Nada es más suave y fluido que el agua, sin embargo, nada se le resiste”. El agua toma la forma de los obstáculos, los abraza, cuando he tenido obstáculos tú los has abrazado conmigo, me has acompañado a lo largo de este camino, me has dado de beber cuando he tenido sed, me has refrescado cuando he estado fatigado. El agua alimenta a la vida y tú alimentas mi vida. Amor mío, gracias por estar a mi lado. Gracias por ser mi agua.

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Summary.

Phytochrome Interacting Factors (PIFs) play a key role in hypocotyl elongation by integrating light, clock and hormonal signals to mediate rhythmic growth (Leivar and Quail 2011). These transcription factors bind G-box (CACGTG) and E-box (CANNTG) elements in the promoters of a diverse set of genes with auxin-related and cell wall-remodeling function and activate their expression (Castillon, Shen et al. 2007). Increased expression of these genes contributes to loosen cell wall, an essential step for elongation growth. Transcriptional activity of these factors is tightly regulated, to prevent excessive growth. Light is the most prominent cue regulating their activity, as it induces rapid destabilization of these factors, via interaction with photoactivated PHYB. This promotes PIFs phosphorylation and marks these factors for proteasomal degradation (Castillon, Shen et al. 2007). GA signaling plays an additional in PIFs regulation, with DELLAs being reported to bind the bHLH region of these factors to block DNA recognition ability (de Lucas, Daviere et al. 2008). However, how light and GA signals are coordinated to restrain PIFs activity to the end of the night, hence determining the rhythmic growth pattern observed under SD photoperiodic conditions remains largely unclear.

Interestingly, in a yeast two hybrid screen using the GAI protein as bait, the clock ELF3 protein was uncovered as a new interacting partner of these repressors. This protein, exclusive of plants, plays an important role in the circadian clock by feed-back loop regulating different core components of the clock. Rhythmic seedlings growth depends on clock function, as mutants in the clock core components display an elongated phenotype associated to arrhythmic growth (Nozue, Covington et al. 2007; Niwa, Yamashino et al. 2009). However, the molecular mechanisms underlying this regulation poorly understood at the time this work was initiated. The finding that DELLAs interact with the clock-related ELF3 protein was very rewarding, as pointed to a possible mechanism for rhythmic growth regulation, thus connecting the central oscillator to elongation growth. The elongated phenotype of *elf3* mutants actually points to a role of this protein in growth repression, indicating that ELF3 might contribute to DELLA stabilization or to the repression of PIFs activity.

In this work we have found that mutations in the *pif4pif5* genes suppress the elongated phenotype of *elf3-8* mutants, these factors therefore acting downstream of ELF3. Over-expression of the ELF3 protein, in turn, suppresses the elongated phenotype of *PIF4*ox lines, indicating that ELF3 functions as a negative regulator of these factors.

Expression of the *PIF4* was found to be misregulated in *elf3-8* seedlings, with increased levels of these transcripts observed at early night. During this interval of the day, growth is usually restricted (Nozue, Covington et al. 2007), elevated expression of these factors hence explaining the tall hypocotyl and arrhythmic growth phenotypes of *elf3-8* mutants. Although ELF3 was recently reported to recruit the ELF4 and LUX proteins into the so called evening complex (EC) (Nusinow, Helfer et al. 2011), that represses *PIF4/PIF5* expression at dusk, we observed that the PIF4 and PIF5 factors directly interact with the ELF3 protein. This interaction is mediated by the C-ELF3 and bHLH domains of these proteins and consistent with ELF3 interaction with the PIF4 DNA recognition domain we established that ELF3 inhibits transcriptional activity of this factor during the day. Although PIFs are widely accepted to be stable only in the dark, we obtained evidence showing that the PIF4 protein starts to accumulate late in the night to peak during the day, although this protein is transcriptionally active only during late night. ELF3 contributes to inhibit PIF4/PIF5 activity during the day, this protein not only repressing PIFs activity as part of the EC complex but also via protein-protein interaction with these factors. Moreover, we observed that PIF4 modulates ELF3 stability, possibly through PHYB-mediated stabilization of this protein. ELF3 is actually reduced in *PIF4*ox lines and levels of this protein are elevated in the *pif*Q quadruple mutant, evidencing a complex regulation of these proteins and raising the possibility that PIF4/PIF5 activity feeds-back into the clock by modulating activity of the ELF3 negative loop. Although effects of this regulation were found to be mild in seedlings, adult plants over-expressing the PIF4 and ELF3 proteins show an intermediate phenotype to ELF3 and PIF4 over-expressers. These seedlings show increased vigor and a prolonged life cycle and are not delayed in floral transition, thus raising a potential agronomic interest of this interaction.

On the other hand, by further analyzing the interaction of ELF3 and DELLAs we have shown that this interaction is mediated by the M-ELF3 domain of ELF3 and the first leucine heptad repeat of DELLAs. Interestingly, M-ELF3 related domains were found to be present also in additional proteins and to be sufficient for DELLA interaction, hence identifying this region as a novel DELLA-interaction domain. As ELF3 had been reported to function as an adapter for COP1 interaction (Yu, Rubio et al. 2008), we analyzed levels of the RGA protein in these two mutant backgrounds. We actually observed that this repressor is strongly stabilized in these mutants in the dark. However, whereas high levels of the RGA protein can be detected in the *elf3-8* mutant, independently of the light-dark cycling conditions, RGA accumulates in *cop1-4*

seedlings after transition to dark, but activity of this E3 ligase is required for stabilization of this repressor upon transition to light. Noteworthy, high levels of the RGA repressor in *elf3-8* mutants contradicted the tall phenotype of these mutants. We show that stabilization of this repressor leads to misexpression of GA-signaling and GA-metabolic genes in this mutant background. Moreover, by generating lines expressing the p*RGA*::GFP-RGA construct in these genetic backgrounds, we show that RGA accumulates in all plant organs in *cop1-4* seedlings, but that in *elf3-8* and *phyB* seedlings this repressor mostly accumulates in the cotyledons. Interestingly, a preferential accumulation of the RGA repressor in the hypocotyl is observed in *PHYB*ox plants, with increased levels of the RGA protein correlating with the hypocotyl lengths and cotyledon sizes of these different plants. Thus, ELF3 and PHYB appear to play an important role in modulating RGA levels in the cotyledons and in this manner its expansion, while COP1 would have a more general effect in all the plant. However, further studies will be required to clarify the exact function of these proteins in modulating RGA stability, keeping in mind that ELF3 interacts with the PHYB, COP1 and DELLA proteins, and that COP1 is required for DELLA stabilization upon transition to light. Altogether, these results evidence a novel mechanism restricting action of the DELLA repressors to specific tissues, unraveling a prevalent role of light in the control of stability of these repressors, independently of GA signaling.

Abbreviations.

2-ODDs: 2-oxoglutarate–dependent dioxygenases.

ATHB2: *Arabidopsis thaliana* HOMEOBOX PROTEIN 2.

bHLH: basic helix loop helix.

BiFC: Bimolecular fluorescence complementation.

CAB2: Chlorophyll A/B binding protein 2.

Cb+: Carbenicilin resistance.

CCA1: Circadian Clock Associated 1.

cDNA: Complementary DNA strand.

CHX: Cicloheximide.

Col-0: ecotype Columbia-0.

CO: CONSTANS.

COP1: CONSTITUTIVELY PHOTOMORPHOGENIC 1.

cRL: Continuous red light.

CRY1, -2: Cryptochrome 1 and 2.

DD: Continuous dark.

SLR1: Slender 1.

ELF3: EARLY FLOWERING 3.

ELF4: EARLY FLOWERING 4.

EOD: end of the day.

EC: evening complex formed by ELF3, ELF4 and LUX/NOX.

FAR1: FAR RED IMPAIRED RESPONSE 1.

FHY3: FAR RED ELONGATED HYPOCOTYL 3.

FKF: FLAVIN BINDING KELCH REPEAT.

FR: Far red.

FT: FLOWERING LOCUS T.

GA: gibberellin.

GAI: GA INSENSITIVE

GA20ox1-5: GA 20-OXIDASES.

GA2ox1-4,6-8: GA 2-OXIDASES.

GA3ox1-4: GA 3 OXIDASES.

GID1: GA-INSENSITIVE DWARF1.

GGDP: geranyl geranyl diphosphate.

GI: GIGANTEA.

HC: Heat-Cold cycles.

HFR1: LONG HYPOCOTYL IN FAR RED.

HH: Continuous temperature.

HY5: ELONGATED HYPOCOTYL.

IAA19: INDOLE-3-ACETIC ACID INDUCIBLE 19.

Kan+: Kanamycin resistance.

LD: Long day.

Ler: ecotype Landsberg erecta.

LHY: LATE ELONGATED HYPOCOTYL.

LKP2: LOV KELCH PROTEIN 2.

LL: Continuous light.

LOV: Light oxygen voltage.

LUC: Luciferase.

LUX: LUX ARRYTHMO.

NOX: MYB transcription factor highly similar to LUX.

NLS: nuclear localization signal.

Pfr: far-red light absorbing form of the phytochrome.

PHOT1. -2: PHOTOTROPINS.

PHYA-E: PHYTOCHROMES.

PIF 1-7: PHYTOCHROME INTERACTING FACTORS.

PIL1: PHYTOCHROME INTERACTING FACTOR3-LIKE1.

Pr: red light absorbing form of the phytochrome.

PRR9,-7,-5,-3,-1: PSEUDO-RESPONSE REGULATOR proteins.

qPCR: quantitative polymerase chain reaction.

QTL: Quantitative trait loci.

RGA: REPRESSOR OF *ga1.3.*

RGL1: RGA like 1.

RGL2: RGA like 2.

RGL3: RGA like 3.

RVE8: REVEILLE8.

RL: Red light.

RPT5: 26S PROTEASOME AAA-ATPASE SUBUNIT.

SAS: Shade avoidance syndrome.

SCF^{SLY1}: E3 Ubiquitin ligase complex (Skip1, Cullin, F-box).

SD: Short day.

EL1: SERINE/THREONINE CASEIN KINASE I.

SLY1: SLEEPY1 F-box protein.

Spe+: Spectinomycin resistance.

SPY: SPINDLY O-GlcNAc transferase.

SPT: SPATULA.

TOC1: TIMING OF CAB EXPRESSION 1, also called PRR1.

UVR8: UVB-RESISTANCE .8

WL: Continuous white light.

XTR7: XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7.

ZT: *Zeitgeber* time or lights-on signal.

ZTL: ZEITLUPE.

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Introduction.

Plants are sessile organisms and as such have evolved sophisticated mechanisms that allow them acclimating to environmental changes and synchronizing these responses with their own developmental programs, controlling vegetative growth and reproductive transition. Complexity of these responses aids surviving harsh external conditions and also anticipating changes in ambient temperature, day length and the quality of incident light, or even pest and herbivore outbreak. A central mechanism to these responses is the internal clock which, by sensing variation of these external cues in successive days, informs the plant on the seasonal time of the year. Plants are also endowed of enormous plasticity that enables them to cope with unpredictable situations such as storms, flooding, drought or niche competition that compromise their fitness. Ability to respond to these unforeseen conditions relies on additional mechanisms, which often share some common elements with daily regulated responses, and thus are also wired to the clock. Main environmental inputs to these pathways are light, temperature, nutrient availability, and defense to pathogen attack. These external cues are perceived by different families of receptors whose activation leads to important changes in plant hormone homeostasis and in gene expression. Modulation of these signaling cascades by the clock determines the diurnal timing and final extent of the triggered physiological responses. Thus, understanding the mechanisms underlying synchronization of these pathways by the clock to shape plant growth and final architecture is a topic of outstanding interest in plant research. Knowledge gained in the model plant *Arabidopsis* shall actually be pivotal to future biotechnological approaches aimed to the selection of more tolerant crops, able to produce stable yields under changing environments. In this work we aimed at understanding the gating effects of the endogenous clock on the cell elongation response mediated by the PIFs and at establishing how the clock controls inhibition of these transcriptional regulators by the DELLA proteins, a group of GRAS-related repressors with a central role in GA signaling.

The light pathway.

Light is the main source of energy for photosynthesis and also is a critical informational cue on the environment in which the plant grows. Thus, almost every developmental decision during the plant's life, from germination to floral transition, is modulated by light. Plants have evolved several types of photoreceptors perceiving the whole

sunlight spectrum from 280 nm ultraviolet light to the 800 nm far-red light (Figure 1). Light absorption by these photoreceptors triggers changes in the levels of expression of nearly 1/3 of the genes, in a process called photomorphogenesis (Figure 1) that contributes to shape plant architecture and provides plasticity to changing environmental conditions. Several photoreceptors are involved in the rapid inhibition of hypocotyl growth of dark-germinated seedlings during de-etiolation, with a most prevalent role in this response being exerted by the red light receptors phytochromes. These photoreceptors play also a central role in the control of petiole and stem elongation in the adult plant, in the so called shade avoidance response (Casal 2012).

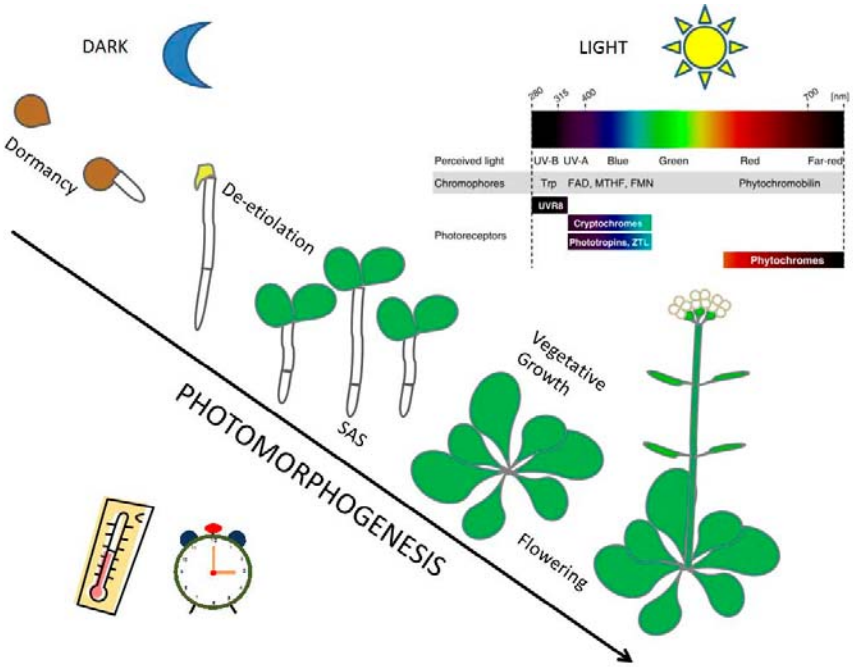

Figure 1. **Plant photoreceptors and photomorphogenesis.** Plants use distinct types of photoreceptors to perceive sunlight covering most wavelengths of the spectrum. These photoreceptors trigger multiple developmental processes, from germination to flowering transition, in a process called photomorphogenesis. These photomorphogenic responses are controlled by external cues such as temperature, light quality and day length. Information provided by these environmental cues is used to entrain the endogenous clock that further synchronizes these responses to maximize plant fitness. Illustration adapted from (Kami, Lorrain et al. 2010).

Noteworthy, similar phytochrome transcriptional cascades modulate hypocotyl elongation in young seedlings and the response to shade in adult plants, these two light-mediated responses being more related than originally thought. For simplicity, in this work we will only focus in the regulation of seedling hypocotyl growth in response to red light.

Plant photoreceptors.

UV-B light.

For short wavelength light as ultraviolet-B (UV-B), growth inhibition is mediated by the UVR8 receptor (Favory, Stec et al. 2009), which is widely and constitutively expressed in all tissues of the plant. This photoreceptor accumulates in a dimeric form that perceives UV-B by a tryptophan-based mechanism. UV-B light absorption induces instant monomerization of the photoreceptor and interaction with the E3 ligase COP1 (*CONSTITUTIVELY PHOTOMORPHOGENIC 1*) to regulate gene expression (Rizzini, Favory et al. 2011). The UVR8 protein monomers revert rapidly into their dimeric form in darkness (Heilmann and Jenkins 2013), a condition that favors hypocotyl growth.

UV-A/Blue light.

Light in the UV-A/Blue wavelength (320-500 nm) is perceived by three classes of photosensory receptors: the cryptochromes (CRY1, CRY2), the LOV/F-box/Kelch domain proteins (ZTL, FKF, and LKP2) and the phototropins (PHOT1, PHOT2) (Christie 2007; Yu, Liu et al. 2010). These proteins in addition to limit plant hypocotyl growth (Kiyosue and Wada 2000; Nelson, Lasswell et al. 2000; Schultz, Kiyosue et al. 2001; Kim, Hicks et al. 2005; Christie 2007), play important roles in modulating circadian rhythms and flowering time (Baudry, Ito et al. 2010; Yu, Liu et al. 2010).

As blue light is perceived by the chromophore of cryptochromes, these photoreceptors change their conformation and bind COP1 and the COP1-interacting protein SPA1 (SUPPRESSOR OF PHYTOCHROME A) to suppress COP1 activity (Yu, Liu et al. 2010). By this means, photoactivated cryptochromes displace interaction of COP1 with the bZIP factor *ELONGATED HYPOCOTYL* (HY5) and cause accumulation of this factor, hence mediating expression of light activated genes and the inhibition of hypocotyl growth (Wang, Ma et al. 2001; Yang, Tang et al. 2001). Other photomorphogenic responses modulated by cryptochromes are the stimulation of cotyledon expansion, the differentiation of chloroplasts and the photoperiodic control of flowering among others (Yu, Liu et al. 2010).

The LOV/F-box/Kelch domain proteins and phototropins absorb blue light by their LOV domains and flavin-mononucleotide (FMN) chromophore, inducing a reversible conformational change that activates their signaling cascades through the E3 ligase (ZTL, FKF and LKP3) or serine/threonine kinase activity (phot1, phot2) associated to these proteins (Christie 2007; Ito, Song et al. 2012). While the LOV/F-box/kelch family

of proteins perceive blue light inputs and transduce this signal to photoperiodic control of flowering and circadian rhythm entrainment (Ito, Song et al. 2012), phototropins mediate phototropism and stomatal opening responses to blue light (Christie 2007).

Far-Red/Red light.

Light in the red/ far-red (600-800 nm) wavelengths is perceived in *Arabidopsis* by a set of five phytochromes (PHYA-PHYE), of which PHYA is light labile, while the rest, PHYB-E, are light stable (Franklin and Quail 2010). Phytochromes structure and regulatory function have been extensively studied and reviewed in the literature, although signal transduction by these photoreceptors is not yet fully understood. Phytochromes are soluble chromophore-bound proteins with two separate domains: an N-terminal domain (with subdomains: NTE N-terminal extension, PAS Per-Arnt-Sim, GAF cGMP binding, and PHY) and a C-terminal domain (PRD PAS related domain and HKRD histidine kinase related domain) linked by a flexible hinge (Li, Li et al. 2011).

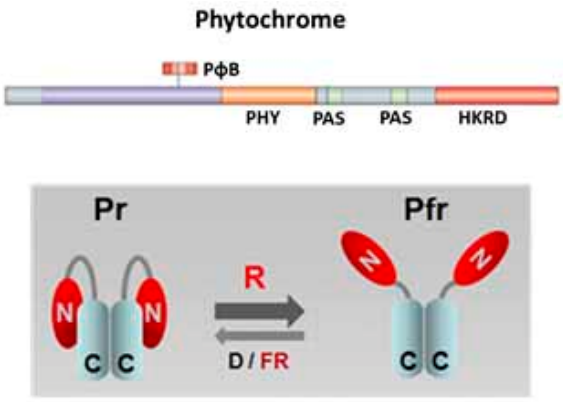

Figure 2. Phytochromes. *Arabidopsis* has a set of five phytochromes (PHYA-E) (Franklin and Quail 2010), or soluble chromophore-bound proteins with two distinct domains: an N-terminal domain comprised by the NTE (N-terminal extension), PAS (Per-Arnt-Sim), GAF and PHY regions, and a C-terminal domain comprised by the PRD (PAS related domain) and HKRD (histidine kinase-related domain) regions, connected by a flexible hinge (Li, Li et al. 2011). These proteins form dimeric structures and depending on light, they oscillate between two conformational states: an inactive red light absorbing (Pr) form and the active far-red absorbing (Pfr) form (Sharrock and Clack 2004). Illustration adapted from (Jiao, Lau et al. 2007; Ueguchi-Tanaka, Hirano et al. 2008; Harberd, Belfield et al. 2009; Jang, Henriques et al. 2010).

These dimeric proteins oscillate, depending on light irradiation, between two conformational states: an inactive red light (Pr) absorbing form and a far-red light absorbing (Pfr) or active form (Rockwell, Su et al. 2006). Reversible conversion

between these two forms depends on the chromophore group attached to the N-terminal GAF region, and is a distinctive trait of phytochrome signaling (Nagatani 2010; Li, Li et al. 2011). All steps for chromophore (phytochromobillin PΦB) synthesis take place in the plastids, while assembly of the new holo-phytochrome in the P_r form occurs in the cytosol (Li, Li et al. 2011). Upon light exposure, the bilin chromophore group changes its conformation and subsequently that of the attached protein, photoactivated phytochromes being then translocated into the nucleus, where they localize in speckles (Kircher, Kozma-Bognar et al. 1999)

Both N-terminal and C-terminal domains of the protein are required for nuclear translocation, with these domains involved respectively in light perception and dimerization (Usami, Matsushita et al. 2007). Functional studies revealed that the PAS and GAF domains are the core of phytochrome signaling (Nagatani 2010; Li, Li et al. 2011) and that nuclear translocation of the protein is critical for its biological function (Huq, Al-Sady et al. 2003), activation of these photoreceptors being correlated with massive changes in gene expression (Tepperman, Hudson et al. 2004; Tepperman, Hwang et al. 2006). Interestingly, not all Pfr phytochrome is translocated into the nucleus upon light exposure (Rausenberger, Hussong et al. 2010), with a pool of active molecules remaining in the cytosol, where there were reported to play a role in gravitropism (Rosler, Klein et al. 2007) and the regulation of mRNA translation (Paik, Yang et al. 2012).

Phytochromes have overlapping but also distinctive roles, depending on the light environment. PHYA is involved in far-red light signaling, while the rest of phytochromes modulate responses to red light. PHYB exerts a main role in red light, compared to the minor roles of PHYC-E (Kami, Lorrain et al. 2010). PHYB and PHYD were reported to form homodimers and heterodimers, whereas PHYC and PHYE obligatorily heterodimerize with the rest of light stable phys (PHYB-E) (Sharrock and Clack 2004; Clack, Shokry et al. 2009). By contrast, the light unstable PHYA exists only as a homodimer (Clack, Shokry et al. 2009). Isolation of quintuple phytochrome mutants showed that these photoreceptors are not necessary to complete the plant's life cycle, these mutant seedlings being fully viable despite lacking the transcriptomic response to red light (Strasser, Sanchez-Lamas et al. 2010; Hu, Franklin et al. 2013). These *phy*-less mutants synthesize chlorophyll and differentiate functional chloroplasts in red light, yet require elevated fluence rates for survival. These plants show defects in several developmental transitions and for instance, require exogenous application of

gibberellins (GA) to germinate or loss-of-function alleles of the *FT* (*Flowering Locus T*) locus to prevent precocious flowering. These results indicate that no other photoreceptors are involved in seed dormancy break and that phytochrome-mediated promotion of GA synthesis is essential for germination (Strasser, Sanchez-Lamas et al. 2010; Hu, Franklin et al. 2013). Also, the quintuple mutant flowers extremely early regardless of the photoperiod, evidencing the importance of light quality in the control of flowering time (Strasser, Sanchez-Lamas et al. 2010; Hu, Franklin et al. 2013). Interestingly, circadian oscillation of leaf movement in these mutants is robust in white light, but is hard to detect under red light (Strasser, Sanchez-Lamas et al. 2010), although under these conditions the *CCA1* (*Circadian Clock Associated 1*) gene, encoding a component of the central clock, still shows rhythmic oscillation but with reduced amplitude. Therefore, phytochromes function as important inputs to the clock, although these photoreceptors are not essential components of the central oscillator (Hu, Franklin et al. 2013).

Phytochrome signaling: the PHYTOCHROME-INTERACTING FACTORS.

Light responses mediated by phytochromes have been widely associated to the direct regulatory effects of these photoreceptors on different transcriptional regulators, and mainly a family of basic helix loop helix (bHLH) transcription factors, known as *PHYTOCHROME INTERACTING FACTORS* (PIFs) (Li, Li et al. 2011) (Figure 3). These factors have been implicated in most photomorphogenetic responses and play a central role in plant development (Castillon, Shen et al. 2007; Leivar and Quail 2011). PIFs belong to the subfamily 15 of the Arabidopsis bHLH family and several of these regulators share an APB (*active PHYB binding*) phytochrome B binding domain (Khanna, Huq et al. 2004; Leivar and Quail 2011) (Figure 3) that drives rapid destabilization of these proteins upon PHYB interaction in the light. PIFs bind conserved G-box (CACGTG) and E-box (CANNTG) elements in the promoters of their regulated genes (Castillon, Shen et al. 2007; Hornitschek, Kohnen et al. 2012) and play a main role in the massive changes in gene expression observed upon activation of PHYB signaling (Quail 2002; Jiao, Lau et al. 2007; Li, Li et al. 2011). Although first identified as light repressors, these factors were later shown to function as promoters of elongation growth and, with exception of PIF7, to be unstable in light. Destabilization of these factors depends on PHYB-mediated phosphorylation, what marks them for degradation by the ubiquitin 26S-proteasomal machinery (Bauer, Viczian et al. 2004; Al-Sady, Ni et al. 2006; Shen, Khanna et al. 2007; Leivar, Monte et al. 2008; Shen, Zhu et al. 2008). Thus, it is now accepted that PIFs sustain etiolated growth and that they

are negatively regulated by phytochromes (Monte, Al-Sady et al. 2007), although the specific kinase and E3 ligase activities implicated in PHYB-mediated degradation of these nuclear factors remain to be identified.

PIFs exist as dimeric molecules and have overlapping but also distinctive roles in plant development (Shin, Kim et al. 2009). These factors are able to heterodimerize (Castillon, Shen et al. 2007), and have additive effects on several photomorphogenic responses (Shin, Kim et al. 2009). Functional analyses of double, triple and quadruple PIF mutants showed that PIF3 and PIF4 exert a predominant role in hypocotyl length control, although PIF1 and PIF5 have also redundant functions, as highlighted by the constitutive photomorphogenic phenotype of quadruple *pifq (pif1pif3pif4pif5)* mutant seedlings, and its altered gene expression profile (Shin, Kim et al. 2009). Studies concerning regulation of hypocotyl growth have thus been mostly directed to the dissection of PIF3 and PIF4 function, and they revealed a complex molecular interaction between these light signaling components and PHYB. Interaction of PIFs with PHYB is light dependent, these factors showing binding affinity only for the active Pfr form (Ni, Tepperman et al. 1999; Huq and Quail 2002). The N-terminal domain of the PHYB photoreceptor is the responsible of this light-dependent selective interaction, although deletion analyses showed that the C-terminal domain displays constitutive PIF binding affinity. This suggests that light-induced structural changes in the photoreceptor are important for enhancing the binding affinity of these proteins (Zhu, Tepperman et al. 2000; Khanna, Huq et al. 2004). Although the conserved APB domain in the PIF factors mediate PHYB interaction, PHYB binding affinities differ among PIFs. This indicates that additional regions contribute to this interaction as exemplified by PIL1 (*Phytochrome Interacting Factor3-Like1*), where a deletion containing the APB domain binds PHYB, but the full length protein is unable to interact with this photoreceptor (Khanna, Huq et al. 2004) (Figure 3).

PIFs were found to be phosphorylated (Al-Sady, Ni et al. 2006) and co-localize with the translocated Pfr form of PHYB in nuclear bodies, within minutes of exposure to light (Huq and Quail 2002; Kim, Yi et al. 2003; Bauer, Viczian et al. 2004; Fujimori, Yamashino et al. 2004; Oh, Kim et al. 2004; Leivar, Monte et al. 2008). These nuclear structures are likely to correspond to sites of PIFs degradation, since the multiubiquitin-binding protein HEMERA, which was reported to modulate PIF1 and PIF3 levels, co-localizes into these nuclear bodies (Chen, Galvao et al. 2010) (Figure 4a).

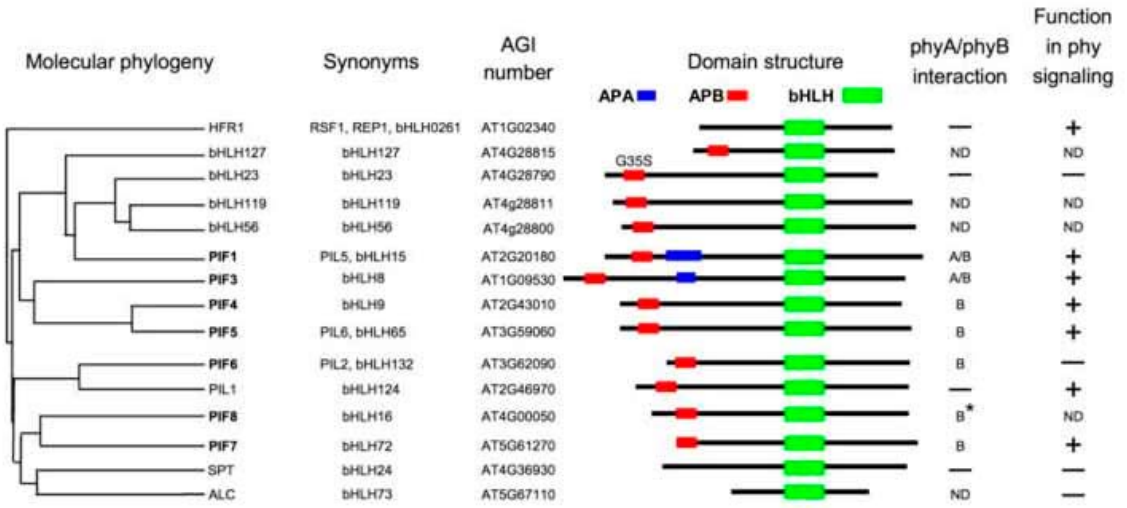

Figure 3. Phytochrome Interacting Factors. PIFs belong to the subfamily 15 of the *Arabidopsis* bHLH family of transcription factors and most of these regulators share an APB (*active PHYB binding*) phytochrome B binding domain. PIF1 and PIF3 show an additional APA (*active PHYA binding*) domain, involved in phytochrome A interaction, and are also regulated by this light-labile phytochrome (Khanna, Huq et al. 2004; Leivar and Quail 2011). These factors recognize G- and E-box elements in the promoters of their regulated genes (Hornitschek, Kohnen et al. 2012; Oh, Zhu et al. 2012). Figure has been adapted from (Leivar and Quail 2011).

Hence, both co-localization and degradation studies support the notion that PHYB represses growth elongation by negatively regulating this family of regulators with a photomorphogenesis repressor function. In fact, targeted degradation of key regulators with a negative regulatory function is a widespread mechanism of modulation in plant development (Huq 2006), being by now widely accepted that light-dependent PHYB signaling triggers PIFs degradation, via direct interaction with these light signaling repressors (Figure 4a). Although several of the morphological changes that result from seedling de-etiolation are only observed after several hours of exposure to light, this developmental switch is triggered by degradation of PIFs. Depending on the duration of light exposure, different sets of genes were found to be differentially expressed, with genes encoding transcription factors showing an early response, while photosynthesis-related genes are regulated at later stages. Moreover, PHYA seems to play a main role early after light irradiation, while PHYB is the predominant photoreceptor after longer exposure to light. Thus, differentially expressed genes after longer exposure to light may not be representative of the transcriptional changes induced during the early de-etiolation response (Tepperman, Hudson et al. 2004; Tepperman, Hwang et al. 2006;

Monte, Al-Sady et al. 2007).

Interestingly, in the light, a residual pool of PIFs still remains in the nucleus (Shen, Moon et al. 2005; Nozue, Covington et al. 2007; Soy, Leivar et al. 2012), which suggests a dual function of these factors in light regulation: a short term regulatory role in early de-etiolation and a longer–term role likely to be implicated in other light-regulated responses. While PHYB-mediated destabilization explains function of these factors in de-etiolation, their role in long-term light signaling is less understood. Recent findings have shed some light into this additional function, as showed that PIFs contribute to regulate PHYB levels (Leivar, Monte et al. 2008; Leivar, Monte et al. 2012; Leivar, Tepperman et al. 2012), as they promote ubiquitination of this photoreceptor by the E3 ligase COP1 (Jang, Henriques et al. 2010) (Figure 4a). This suggests that long-term PIFs modulated responses are mediated by a negative feedback regulatory loop. Supportive evidence for this model has been at least obtained for PIF4, since expression of a mutated form of this factor, lacking the APB PHYB interaction domain, is unable to restore the phenotype of *slr2* mutants, with a loss-of- function mutation in the PIF4 gene (Khanna, Huq et al. 2004). However, further research will be required to demonstrate that deletion of the APB domain does not impair PIF4 transcriptional activity and to establish that this domain is in fact required for negative modulation of PHYB levels, since interaction of both proteins might be essential for this activity.

Nuclear translocation of phytochromes, on the other hand, has been reported to depend on other facilitator proteins (Genoud, Schweizer et al. 2008; Pfeiffer, Nagel et al. 2012) as PIFs, that contribute to the nuclear import of PHYB (Pfeiffer, Nagel et al. 2012). Consistent with this function, nuclear translocation of PHYB is compromised in *pif* mutants, COP1-dependent turnover of PHYB being compromised in these seedlings, which leads to elevated levels of PHYB. Regulation of PIFs and PHYB is thus mutually interconnected, this intricate regulation providing a very robust mechanism for cell elongation control, at the time that enables a fast and sensitive response to external light signals. Based on these findings, PIFs are now accepted to be central hubs for integration of light and environmental signals, these factors playing an essential role in orchestrating plant growth and development in response to external cues (Lucyshyn and Wigge 2009) (Figure 4b).

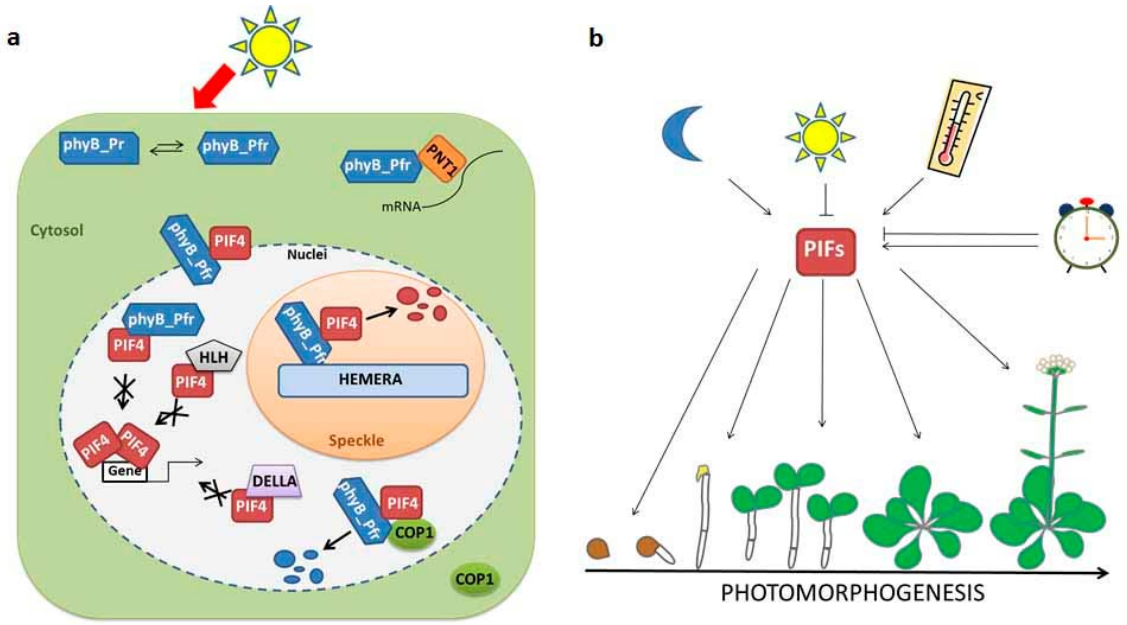

Figure 4. PIFs pivotal role in plant development. a) Light modulates nuclear accumulation of these transcriptional regulators. Upon light absorption PHYB is translocated into the nucleus, where it co-localizes with PIFs. Interaction with PHYB promotes phosphorylation of these factors and marks them for proteasomal degradation (Chen, Galvao et al. 2010; Jang, Henriques et al. 2010). PIFs facilitate nuclear import of PHYB and contribute to the turnover of this photoreceptor. Complex formation with PHYB, the DELLA repressors, and atypical HLH inhibits PIFs transcriptional activity by blocking their DNA binding ability (de Lucas, Daviere et al. 2008; Hao, Oh et al. 2012; Park, Park et al. 2012). b) PIFs function as main integrators of different environmental signals and play a pivotal role in photomorphogenesis by sustaining etiolated growth and modulating response to shade and floral transition (Lucyshyn and Wigge 2009; Leivar and Quail 2011).

In addition to be destabilized by PHYB, PIFs transcriptional activity has been also shown to be repressed by heterodimerization with atypical HLH factors induced in response to shade (Hornitschek, Lorrain et al. 2009; Hao, Oh et al. 2012), by interaction with PHYB (Park, Park et al. 2012) and by binding to the DELLAs, a set of repressors of GA signaling that bind the bHLH DNA recognition domain of these factors (de Lucas, Daviere et al. 2008; Feng, Martinez et al. 2008) (Figure 4a). This complexity of regulation suggests that in addition to light signaling, PIFs may also function as nodes for cross-talk with other signaling pathways. Light is still the most prevalent regulatory cue for these factors, as it triggers PIFs degradation. However, in dark or far-red enriched stabilizing conditions, PIFs were shown to bind as well other central regulators like the DELLAs and thus to function as integrators endogenous signaling

pathways to coordinate plant growth in response to light and hormonal signals (de Lucas, Daviere et al. 2008). Cross-regulation of these pathways is likely to be highly relevant in nature, where an orchestration of the responses to environment with the own plant developmental programs is essential to safeguard fitness and warrant survival to adverse conditions. In the field, plants are actually subjected to notable changes in day length and temperature as seasons progress, as well as to important changes in light quality during the day. While at the height of the day, incident light covers the whole light spectrum, as the sun rises or descends the horizon, the path length of solar radiation increases and atmospheric reflection and refraction, along with absorption by ozone, modifies the predominance of certain light wavelengths (Kami, Lorrain et al. 2010). During twilight, the light spectrum changes to deep blue, whereas the starlight spectrum is red. Moon light may also modulate some responses, but these have not been studied in detail. Also, quality of incident light is different in an open field than under vegetation, as filtration through a canopy causes a strong reduction in blue and red light, due to light absorption by the photosynthetic pigments of the leaves (Keller, Jaillais et al. 2011). Therefore, a complex sum of regulatory cascades is likely in nature to modulate plant growth. Convergence of these regulatory networks into common signaling intermediates as the PIF factors, that function as main cross-talk regulatory hubs, enables proper orchestration of these responses and is essential to fine tune modulate gene expression to highly variable environmental conditions. Extensive cross-talk between the light and hormonal signaling pathways is highlighted by the interaction of PIFs with DELLAs, inhibition of PIFs transcriptional activity by these repressors actually featuring a protein regulatory cascade that plays a prevalent role in light and GA signaling (de Lucas, Daviere et al. 2008).

The gibberellin pathway.

GA metabolism.

Gibberellins (GA) are a large family of diterpenoid hormones, including an important number of inactive biosynthetic precursors and catabolic products, along with the biologically active GA_1, GA_3, GA_7 and GA_4 molecules. GA metabolism and signaling has been widely studied in different plant species (Yamaguchi 2008; Sun 2011; Wang and Deng 2011; Hedden and Thomas 2012), with increased levels of these hormones associated to seed germination, hypocotyl growth, leaf expansion, floral transition and flower and fruit development, and shown to affect most aspects of plant development. Biosynthesis of GAs can be divided in three main steps (Olszewski, Sun et al. 2002): i)

biosynthesis of *ent*-kaurene in proplastids; ii) conversion of *ent*-kaurene to GA_{12}, via microsomal cytochrome P450 monooxygenases; and iii) formation of C20- and C19-GAs in the cytoplasm (Figure 5a). The first biosynthetic steps from geranyl geranyl diphosphate (GGDP) to *ent*-kaurene are catalyzed by two terpene cyclases, CPS and KS, and are the first committed steps for GA biosynthesis (Yamaguchi 2008). Mutations in either of these two genes (*ga1* and *ga2*, respectively) cause important developmental defects like severe dwarfism, reduced fertility, impaired germination and loss of apical dominance (Silverstone, Mak et al. 1997; Yamaguchi 2008). Isolation of the *Arabidopsis ga1-3* mutant, with a loss-of-function mutation in the CPS *GA1* gene, has actually been instrumental to determine the biological function of these hormones and to dissect their metabolic and signaling pathways. Conversion of *ent*-kaurene to GA_{12}, the common precursor of bioactive GAs in all plant species (Hedden and Thomas 2012), is catalyzed by two cytochrome P450 monooxigenases (KO and KAO), localized respectively at the outer membrane of plastids and the microsomal fraction of the endoplasmic reticulum (Yamaguchi 2008). GA_{12} is then oxidated in the cytosol by a group of 2-oxoglutarate–dependent dioxygenases (2ODDs), to yield the bioactive compounds, GA_1, GA_3, GA_4 and GA_7 (Yamaguchi 2008; Hedden and Thomas 2012). The first of these oxidations results in loss of one carbon and leads to the conversion of C-20 GAs into C-19 GAs, in a reaction catalyzed by GA 20-oxidases, a group of enzymes that *Arabidopsis* is encoded by a family of five genes (*GA20ox1* to *-5*) (Yamaguchi 2008). *GA20ox1*, *-2, -3* correspond to the dominant paralogs, and code for enzymes with similar activities, but differing in their expression patterns. The roles of *GA20ox4* and *-5* are much minor, with the GA20ox5 enzyme catalyzing only part of the oxidizing steps that lead from GA_{12} to GA_9 conversion (Plackett, Powers et al. 2012). Selection of double and triple loss of function mutations for these genes revealed that GA20ox1, -2 and -3 are the major enzymes and that loss of function of these three genes results in similar developmental defects than the *ga1-3* mutation, indicating a less relevant function of the *GA20ox4* and *-5* genes (Rieu, Ruiz-Rivero et al. 2008; Plackett, Powers et al. 2012). The products of GA20ox oxidation (GA_9 and GA_{20}) are further 3β-hydroxylated by ODD GA3 oxidases, to convert them into bioactive GAs (Hedden and Thomas 2012). In *Arabidopsis*, these enzymes are encoded by a family of four *GA3ox* genes (*GA3ox1* to *-4*), which are differentially regulated but share redundant functions (Hedden, Phillips et al. 2001). Thus, *GA3ox1* and *-2* are expressed in all organs and play a prevalent role in seed germination and vegetative growth, while expression of *GA3ox2* is restricted to the first stages of plant development. In turn,

GA3ox3 and *-4* are specifically expressed in reproductive organs, their function being relevant during this stage of development (Mitchum, Yamaguchi et al. 2006). Thus, the double *ga3ox1 ga3ox2* mutant displays a dwarf phenotype but is fertile, which confirms a distinct role of these genes (Mitchum, Yamaguchi et al. 2006). Since GA3ox enzymes catalyze the final step of GA biosynthesis, their expression has been related to active sites of GA signaling (Hu, Mitchum et al. 2008). Other studies however suggested that GAs are transported (Lofke, Zwiewka et al. 2013; Shani, Weinstain et al. 2013), being at present unclear if only the precursors or final bioactive molecules are mobile, further research being required to clarify which form of these hormones is transported. Bioactive GA levels were also found to be regulated in a tissue and developmental specific manner by negative feedback regulation of the biosynthetic ODD (GA20ox and GA3ox) genes and forward regulation of catabolic enzymes (Yamaguchi 2008). Bioactive GAs, on the other hand, are inactivated by 2β-hydroxylation, in a reaction catalyzed by a set of ODD GA2 oxidase enzymes, encoded in *Arabidopsis* by a family of seven genes (Yamaguchi 2008; Hedden and Thomas 2012). These enzymes were grouped in three classes according to their substrates and phylogenetic relationships. Class I and II use C19-GAs as substrates, while class III catalyzes hydroxylation of C20-GAs. Thus, class I (*GA2ox1,-2,-3*) and II (*GA2ox4,-6*) act over bioactive GAs and their immediate precursors, comprising in *Arabidopsis* the mayor gibberellin inactivation pathway, while class III (*GA2ox7, -8*) are involved at hydroxylation of earlier C20 precursors of the GA pathway (Rieu, Eriksson et al. 2008). Genes encoding the class I and II enzymes are ubiquitously expressed and their transcripts are detected throughout plant development, with bioactive GA levels being remarkably increased in quintuple mutants carrying loss-of-function mutations in all these genes. These mutants show an elongated phenotype that mimics that of GA treated plants, hence pointing to an overlapping and redundant function of these enzymes in GA inactivation (Rieu, Eriksson et al. 2008). In addition to 2β-hydroxylation, bioactive GAs are also inactivated by GA methylation (Varbanova, Yamaguchi et al. 2007) and epoxidation of the C-16,17 double bond (Zhu, Nomura et al. 2006). However, GA-methyl transferase activity of GAMT1 and GAMT2 appears to be restricted to developing seeds (Varbanova, Yamaguchi et al. 2007), whereas 16,17-epoxidation has only been demonstrated in rice (Zhu, Nomura et al. 2006).

GA signaling.

Genetic screens for mutants exhibiting an altered response to these hormones identified the RGA (*repressor of ga1-3*) and GAI (*gibberellin insensitive*) proteins, that

function as negative regulators of GA signaling (Peng, Carol et al. 1997; Silverstone, Ciampaglio et al. 1998). These repressors share a conserved C-terminal domain with the plant specific GRAS family of transcriptional regulators (for GAI RGA And Scarecrow), but display a unique N-terminal domain comprised by the highly conserved Asp-Glu-Leu-Leu-Ala amino acid sequence or DELLA domain, specific to this subfamily of repressor proteins (Hauvermale, Ariizumi et al. 2012). In *Arabidopsis*, three additional homologues (RGL1, RGL2, RGL3) were also identified, which share the highly conserved DELLA domain, a middle VHIID domain and a C-terminal RVER region. Based on this homology, these proteins were designated as DELLAs, further studies showing that they play a central role in GA signaling (Hauvermale, Ariizumi et al. 2012; Sun, Jones et al. 2012). These repressors share a Ser/Thr-rich motif thought to be targeted by regulatory phosphorylation or glycosylation events, a conserved LHR domain involved in protein/protein interaction, a nuclear localization signal (NLS) and a SH2 binding domain, which in the Src oncogene has been shown to function as a pTyr-recognition domain (Wang, Zhu et al. 2009; Hauvermale, Ariizumi et al. 2012; Sun, Jones et al. 2012). Loss-of-function mutations of these genes in the *Arabidopsis global* mutant, rescue completely the dwarf phenotype of *ga1-3* mutants, hence indicating that these proteins play redundant functions in GA signaling (Fleet and Sun 2005). Analysis of the phenotypes of different mutant combinations in these genes evidenced that RGA plays a major role in modulating plant height, although GAI and RGL1 further contribute with smaller effects to this trait. RGA, RGL2, and RGL1 play a dominant role in flowering, whereas *RGL2* has a prominent function in seed germination, along with minor effects of RGA, GAI, and RGL3 (Dill and Sun 2001; King, Moritz et al. 2001; Lee, Cheng et al. 2002; Cheng, Qin et al. 2004; Tyler, Thomas et al. 2004; Cao, Hussain et al. 2005; Piskurewicz and Lopez-Molina 2009). Function of RGL3, in turn, has been associated to biotic stress. Partially divergent functions of these genes appear to result mainly from their dissimilar expression patterns than from actual differences in the encoded proteins (Gallego-Bartolome, Minguet et al. 2010). Other reports have shown that these proteins may exhibit different GA-degradation kinetics (Wang, Zhu et al. 2009) although more accurate studies are required to confirm their differential stability. DELLA repressors are actually rapidly destabilized in response to GA (Silverstone, Jung et al. 2001; Dill, Thomas et al. 2004; Tyler, Thomas et al. 2004), with mutations in the DELLA domain (*rga∆17 and gai-1*) impairing GA-induced degradation and causing a semi-dominant GA-insensitive dwarf phenotype associated to the accumulation of these proteins (Peng, Carol et al. 1997; Dill, Jung et al. 2001). Increased levels of

these repressors are also observed in *ga1-3* seedlings, impaired in GA synthesis, which let to postulate that GAs promote growth by relieving DELLA repression. Identification of the GA receptor was decisive to unveil the mechanism by which GAs signal degradation of these proteins and provided a direct molecular link between these hormones and the DELLAs. Bioactive GAs are perceived by a soluble receptor protein encoded by the *GA-INSENSITIVE DWARF1* (*GID1*) gene (Ueguchi-Tanaka, Ashikari et al. 2005). In *Arabidopsis*, three redundant genes (*GID1a*,*-b* and *–c*) encode this GA receptor activity (Griffiths, Murase et al. 2006; Nakajima, Shimada et al. 2006). Single mutations in these genes lead to a mild phenotype, but loss-of-function mutations in all three genes cause an extremely dwarf phenotype that cannot be rescued by exogenous GA, hence demonstrating that these are the unique receptors for GAs in plants. The more severe dwarf phenotype of *gid1a*/*b*/*c* mutants than that of *ga1-3* seedlings, suggest that these soluble proteins may also function in GA-independent signaling (Griffiths, Murase et al. 2006). GA perception triggers a conformational change in the GID1 protein that promotes binding of the GA-GID1 complex to the conserved DELLA domain of the DELLA proteins (Harberd, Belfield et al. 2009; Sun 2011). Formation of the GA-GID-DELLA ternary complex, in turn, fosters interaction of the C-terminal domain of DELLAs with the F-box SLEEPY1 (SLY1) subunit of the SCF^{SLY1} E3 ubiquitin ligase complex (Skip1, Cullin, F-box), show to provide substrate specificity to this complex. Recruitment of DELLAs to this complex results in the ubiquitination of these repressors and triggers them for degradation by the 26S proteasome system (McGinnis, Thomas et al. 2003; Dill, Thomas et al. 2004; Fu, Richards et al. 2004; Harberd, Belfield et al. 2009). A second F-box protein encoded by SNEEZY, has also been identified in *Arabidopsis* as a modulator of GA signaling. However, SNEEZY appears to be involved in degradation of RGA and GAI, but not in the degradation of RGL2, thus exhibiting a only partially redundant function with SLY1 (Ariizumi, Lawrence et al. 2011). Altogether, these findings demonstrate GAs regulate gene expression by triggering degradation of DELLAs, thus relieving transcriptional inhibition imposed by these repressors. Destabilization of these nuclear proteins results in massive changes in gene expression and finally culminates in elongation growth (Harberd, Belfield et al. 2009). Although similarity with members of the GRAS family of transcriptional regulators let to postulate that DELLAs might inhibit gene expression by directly binding the promoters of their regulated genes, attempts to demonstrate a direct DNA binding ability of these repressors failed to the date. Nevertheless, evidence of direct regulation by DELLA transcriptional activity has been documented on rice and

by yeast two hybrid assays, (Hirano, Kouketu et al. 2012). The suppressive function of the rice DELLA protein SLR1 is dependent on its transcriptional activation activity (Hirano, Kouketu et al. 2012). More recent findings showed that these repressors bind the DNA recognition domain of PIFs to sequester these transcriptional regulators into an inactive complex unable to bind to DNA (de Lucas, Daviere et al. 2008). Thus, it is now widely accepted that changes in gene expression driven by these repressors result from their ability to interact with different families of transcriptional regulators, exhaustive efforts being currently dedicated to the identification of these interaction partners (see for review (Daviere and Achard 2013)).

Light and GA signaling cross-talk.

Several lines of evidence point to a role of the DELLA repressors in the growth restriction imposed by light. The *Arabidopsis tetra* mutant carrying loss-of-function mutations in the RGA, GAI, RGL1 and RGL2 genes showed to be insensitive to light-mediated growth inhibition (Achard, Liao et al. 2007) and also the *gai-1* gain-of-function mutation, that leads to the accumulation of this DELLA, shows impaired elongation of the petioles when irradiated with far-red enriched light (Djakovic-Petrovic, de Wit et al. 2007). Hence, DELLAs seem to play a prominent role in light signaling, these repressors being shown to interact with the bHLH domain of the growth promoting PIF3 and PIF4 factors, to block DNA binding ability of these transcriptional regulators (de Lucas, Daviere et al. 2008; Feng, Martinez et al. 2008). Chromatin immunoprecipitation studies also showed that DELLAs associate to the promoters of some genes (Zentella, Zhang et al. 2007), although binding to DNA is thought to be mediated via complex formation with other DNA binding factors. Expression of stable forms of these repressors under control of a dexamethasone inducible construct, on the other hand, has been shown to result in activation of genes with a role in ABA signaling and the activation of GID1 and the last steps in the GA biosynthetic pathway, hence pointing to a further inhibitory effect of DELLAs on DNA binding proteins that negatively regulate ABA and feed-back GA signaling (Zentella, Zhang et al. 2007). Thus, DELLAs are presumed to promote photomorphogenesis and restrain shade avoidance responses also by modifying GA levels (Achard, Liao et al. 2007; Djakovic-Petrovic, de Wit et al. 2007). Higher levels of these hormones have been actually observed in sunflower (*Helianthus annus*) and cowpea (*Vigna sinensis*) in response to low red/far-red treatments (Martinez-Garcıa Jaime F. 2000; Kurepin, Emery et al. 2007), although in these studies it was not analyzed whether higher GA levels correlate with changes in the biosynthetic or inactivation genes. A direct relationship between levels of

expression of the GA biosynthesis and inactivation genes and GA levels is also not always seen in *Arabidopsis*, due to feed-back regulation of these genes. The *Arabidopsis cry1cry2* mutants, for instance, show increased levels of expression of both the biosynthetic and inactivating enzymes that would explain the increased hypocotyl length of these mutant seedlings, but surprisingly the total GA content is not altered in these plants (Zhao, Yu et al. 2007). Also it remains to be elucidated if rapid degradation of DELLAs depends solely on GA levels or is additionally regulated by GA independent components. In addition to that, GA biosynthetic genes show different tissue-specific and even cell-specific expression patterns, suggesting that local changes in bioactive GAs may not be reflected in studies in which the total plant GA content is analyzed. A mathematical model aided with the root growth dynamics has actually predicted a GA gradient along the root, that would explain the reduced growth of cells from the elongating towards the meristem root tip zone (Band, Ubeda-Tomas et al. 2012). In maize, it has also been established that a gradient in GA levels in the leaf results from differential expression of the GA biosynthetic genes in the division zone and that of catabolic genes in the expansion zone, such a spatial distribution of these genes being critical to control growth and final size of the leaves (Nelissen, Rymen et al. 2012). Recent studies using fluorescently labeled GAs also evidenced a cell specific localization of bioactive GAs in roots, by showing that fluorescence was localized in the nuclei of elongating endodermal cells, which presumably serve as sinks of the bioactive GAs produced by the surrounding tissues. Surprisingly, cells in these tissues store GAs in the vacuoles, hence suggesting an active mechanism of transport that also involves individual cell dynamics (Shani, Weinstain et al. 2013). Such cell specificity is also visible in gravistimulated roots, where an asymmetric GA distribution has been shown to stabilize the PIN-dependent flux of auxins that drives differential elongation of the cells causing root bending (Lofke, Zwiewka et al. 2013). Hence, all these findings suggest a complex cell-specific mechanism of control of bioactive GA synthesis that indicates that overall changes in GA content cannot be directly related with changes in the levels of expression of GA biosynthetic and signaling genes.

Alternative mechanisms of DELLAs regulation.

In addition to GA-dependent destabilization, other regulatory mechanisms have also been postulated to play a role in regulating DELLAs activity. Loss-of-function of the SPINDLY (*SPY*) gene, encoding a O-GlcNAc transferase enzyme, were reported to rescue the dwarf phenotype of GA deficient and *gai-1* insensitive mutants, hence implicating this Ser/Thr glycosylating enzyme in GA signaling. SPY function has

actually been postulated to enhance the inhibitory activity of DELLAs but intriguingly, levels of accumulation of these repressors do not change in *spy* mutants. Thus, the molecular mechanism underlying SPY-mediated growth repression remain elusive (Silverstone, Tseng et al. 2007). An additional post-translational modification reported for these repressors is the phosphorylation of the rice SLR1 protein by casein kinase I (EL1). Phosphorylation of this DELLA protein at Ser/Thr positions has been reported to stabilize this repressor and negatively modulate GA signaling (Dai and Xue 2010). Although it remains unclear whether this modification regulates also *Arabidopsis* DELLAs activity, substitution of a conserved threonine in the RGL2 protein, by a negative charged residue that mimics the phosphorylation state, to yield the $RGL2^{T272D}$ mutation, was found to increase stability of this repressor (Hussain, Cao et al. 2005). Also, a Ser/Thr phosphatase activity was shown to be required in cell-free assays for the destabilization of these repressors (Wang, Zhu et al. 2009), hence suggesting that similar phosphorylation events may modulate *Arabidopsis* DELLAs activity. In addition to these modifications, other regulatory mechanisms independent of degradation may also modulate the repressive activity of DELLAs. The *sly1/gid2* mutants, impaired in function of the $SCF^{SLY/GID2}$ F-box subunit, actually show a much milder phenotype than the *ga1-3* or *gid1a/b/c* mutations, although they accumulate comparable or even higher levels of DELLAs. Intriguingly, the phenotype of these mutants is rescued upon over-expression of the GID1 receptor, but the taller phenotype of these lines is not associated with a concurrent reduction in GA levels (Ariizumi, Murase et al. 2008; Ueguchi-Tanaka, Hirano et al. 2008). Thus, it has been postulated that the soluble GID1 proteins would also play a GA-independent role in modulating DELLAs activity, by sequestering these repressors into an inactive complex (Ariizumi, Murase et al. 2008; Ueguchi-Tanaka, Hirano et al. 2008). Function of these repressors has also been reported to be modulated by protein-protein interaction, as seen for the SCARECROW-LIKE 3 (SCL3) GRAS factor, which positively regulates GA signaling, by antagonizing DELLAs function (Gao, Xiao et al. 2011).

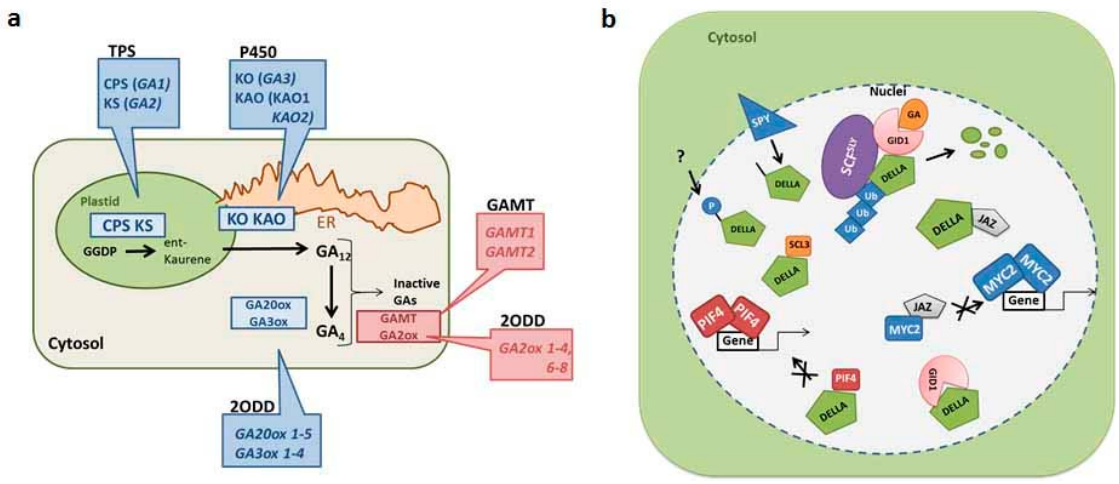

Figure 5. The gibberellin pathway. a) Gibberellin metabolism. Metabolism of these hormones can be divided in the main steps (Olszewski, Sun et al. 2002): 1) biosynthesis of *ent*-kaurene in the proplastids; 2) conversion of *ent*-kaurene to GA_{12} via microsomal cytochrome P450 monooxygenases; and 3) formation of C_{20}- and C_{19}-GAs in the cytoplasm. Balance between bioactive and inactive GAs is mainly regulated by three families of 2ODD (*GA20ox*, *GA3ox* and *GA2ox*) encoding genes. In blue are represented the biosynthetic genes while in pink are the catabolic enzymes. Figure adapted from (Yamaguchi 2008). b) DELLAs regulation and repressive function. DELLAs abundance is mainly modulated in response to GA signaling. Perception of GAs by the GID1 soluble receptor induces a conformational in this protein that results in enhanced binding to the DELLAs (Harberd, Belfield et al. 2009; Sun 2011). Formation of the GA-GID-DELLA complex recruits the SCF^{SLY1}ubiquitin ligase complex that ubiquitinates the DELLAs and targets these repressors for degradation by the 26S proteasome system (Dill, Thomas et al. 2004). Degradation of DELLAs lifts the growth inhibition imposed by these repressors and releases the PIF4 factor (de Lucas, Daviere et. al. 2008). GID1 inhibits DELLA activity in the absence of GAs (Ariizumi, Murase et al. 2008). DELLAs are also modulated by the *O*-GlcNAc transferase activity of SPINDLY (*SPY*) (Silverstone, Tseng et al. 2007; Herrero, Kolmos et al. 2012) and phosphorylation (Dai and Xue 2010; Herrero, Kolmos et al. 2012; Kumar, Lucyshyn et al. 2012). Activity of these repressors is further modulated via protein-protein interaction with the GRAS SCARECROW-LIKE 3 (SCL3) protein (Gao, Xiao et al. 2011; Herrero-Soriano 2011). Interaction of DELLAs with the JAZ proteins releases the MYC2 factor and induces JA-regulated gene expression (Hou, Lee et al. 2010; Herrero, Kolmos et al. 2012).

DELLAs were, in addition, reported to interact with the JAZ proteins, a key family of regulators with a repressive role in jasmonic acid signaling, due to their ability to block MYC2 transcriptional function. While binding of DELLAs to the JAZ proteins, frees the MYC2 factor and enables binding of this transcriptional regulator to conserved elements in the promoters of JA-regulated genes, destabilization of DELLAs favors

JAZ-MYC2 interaction and represses JA-regulated gene expression (Hou, Lee et al. 2010). Hence, it is becoming increasingly clear that DELLAs interact with multiple protein partners which not necessarily need to be directly involved GA signaling. As this interaction often entrains a competitive complex, that blocks DELLA activity or that of the binding partner, a very active area of research is now devoted to the identification of additional interacting partners. Identification of these partners shall be very insightful to understand the biological function of DELLAs and contribute to unveil novel roles of these important regulators (Figure 5b).

GA homeostasis.

Endogenous GA content is regulated through complex negative feedback and feed-forward regulatory loops that control expression of the last biosynthetic steps and catabolic enzymes, leading to a high degree of homeostasis. In this way, reduced GA levels and lower levels of expression of the *GA20ox1* and GA20ox3 genes are observed in the *rga-24 gai-t6* loss-of-function mutant, while increased GA content, and higher levels of expression of the *GA20x1* and *GA3ox1* genes are found in the *gai-1* mutant (Xu, Li et al. 1995; Cowling, Kamiya et al. 1998; Xu, Li et al. 1999). Also, upon exogenous GA application, expression of the *GID1a/b* receptor and *GA20ox1* and *GA3ox1* biosynthetic genes is reduced, while expression of the catabolic *GA2ox1* enzyme and *RGA* is up-regulated, although the newly synthesized RGA protein is degraded (Thomas, Phillips et al. 1999; Dill, Jung et al. 2001; Dill and Sun 2001; Zentella, Zhang et al. 2007). Seedlings expressing the rga∆*17* stable form of RGA, that lacks the DELLA domain, show similar levels of expression of the *GA3ox1* gene as the WT and this gene is not repressed in response to GA, highlighting the role of RGA in feed-back regulation (Dill and Sun 2001). Gene expression analyses of *rga∆17* DEX inducible lines showed, in addition, up-regulated levels of expression of this transcript after 4 hours of induction, thus confirming negative feed-back regulation of this gene (Zentella, Zhang et al. 2007). Mathematical models for GA homeostasis, however, predicted a weaker feed-back regulation of the *GA3ox* genes compared to those encoding the GA20ox biosynthetic enzymes. Feed-back regulation of these genes seems to act synergistically with that regulating *GID1* and *DELLA*s gene expression, these models predicting a crucial role of *GA20ox* gene expression in modulating DELLA protein levels (Middleton, Ubeda-Tomas et al. 2012). Expression of the GA20ox1 and GA3ox1 enzymes, on the other hand is reduced in the *ga2ox* quintuple mutant, but the GID1b receptor is expressed to normal levels (Rieu, Eriksson et al. 2008), independently of other observations showing that this gene is reduced upon GA

application (Griffiths, Murase et al. 2006). On the other hand, all *ga2ox* genes except -3 and -7 are induced by GA treatment (Rieu, Eriksson et al. 2008), whereas among all members of the *GA3ox* gene family, only the *GA3ox1* gene is under negative feedback regulation (Chiang, Hwang et al. 1995; Yamaguchi, Smith et al. 1998; Mitchum, Yamaguchi et al. 2006). A shift from dark to light conditions promotes an accumulation of the DELLAs that coincides with up-regulated levels of expression of *GA2ox1*, and the repression of the *GA3ox1* and *GA20ox1* genes. In opposite, upon shifting from light to dark conditions, DELLAs are destabilized and expression of the *GA2ox1, GA3ox1* and *GA20ox1* genes is reduced (Achard, Liao et al. 2007). Interestingly, none of these changes was found to correlate with changes in DELLA gene expression, and thus they were associated to a regulatory role of phytochromes in modulating bioactive GA levels (Achard, Liao et al. 2007). However, all these analyses were carried out after transitory shifts to light or dark conditions and thus it is unclear whether they are also relevant under photoperiodic conditions, during transition from the day to night periods.

A great deal of evidence indicates that the interplay of GA homeostasis, DELLAs protein stabilization and repression of PIFs transcriptional activity, plays a very relevant role in the control of hypocotyl growth. However, insights pointing to this important mechanism of regulation were obtained under particular experimental conditions, such as the de-etiolation process or from seedlings grown under continuous light. Therefore, these studies neglected the timing of the day in which these regulatory mechanisms become relevant. As seen in the previous sections, function of each of these regulatory components showed to be modulated by light, in addition to the observation that seedlings do not show a constant pattern of growth but, under short days, elongation is restricted to the end of the dark period (Nozue, Covington et al. 2007). Therefore, this signaling network must be subjected to regulation by additional diurnal components that determine when the plant needs to grow. More recent studies are directed to this particular issue, the regulatory mechanisms synchronizing growth elongation with a precise window time of the day hence gaining more interest.

The circadian clock.

As earth rotates on its own axis around the sun, it is constrained to alternating day and night cycles that oscillate within a period of 24 h, or the time required for a complete turn. The Axis of earth rotation is deviated with respect to the perpendicular of rotation around the sun, this axial tilt leading to seasonal changes in day length that depend on the geographical latitude. Close to the equator, intervals of the illuminated and dark

periods are close to 12 hours and do not oscillate much during the year. However, as latitude approaches to the poles, day length is longer in summer and shorter in winter. All earth species have evolved self-sustaining mechanisms which, by sensing diurnal variations in day length and temperature, enables them anticipating to environmental changes associated to seasons progression and synchronize their life cycle with the year season. Periodicity of this self-sustaining mechanisms invariably approximates 24 hours, which is the time required to complete a full turn of the earth (Dunlap Jay C. 2004). Light is an important input to this oscillator (Figure 6) as it serves as the day start signal that entrains this self-sustaining mechanism. Function of this endogenous oscillator, in its turn, impinges on the levels of expression of multiple output genes, as exemplified by the observation that around 40% of the *Arabidopsis* genes show a characteristic diurnal rhythm of oscillation (Covington, Maloof et al. 2008). Noteworthy, this rhythmic pattern of expression is also observed under continuous light or dark conditions, which indicates that a highly robust timekeeping circuit called the "circadian clock" controls levels of expression of these genes (Dunlap Jay C. 2004). This internal clock diurnally regulates a broad range of processes (termed outputs), including hypocotyl growth, leaf movement, stomatal opening, hormone and stress responses, and flowering transition (Chow and Kay 2013). Advances in this area of research have contributed to extensively modify during the past years the accepted models concerning function of this self-sustaining mechanism, being by now made evident by that a highly complex and robust mechanism of regulation drives diurnal oscillation of its core components. The endogenous clock controls, in addition, almost every aspect of the plant's life cycle, from seed dormancy to defense against pathogens and therefore, unveiling the mechanisms controlling this diurnal oscillator, is of capital importance to understand how plants manage to cope and survive to a wide range of adverse environmental conditions (Nakamichi 2011).

The central oscillator.

In *Arabidopsis*, more than 25 independent genes have been identified, whose function has been reported to be required or to contribute to the maintenance of the endogenous circadian clock function (Nakamichi 2011). However, although these factors were proven to provide different levels of complexity to the clock regulation, function of the circadian clock depends on a "central oscillator" comprised by two classes of genes: the morning phased CCA1 and LHY transcription factors, and the evening phased pseudo-response regulator genes (PRR9,-7,-5,-3,-1 and RVE8 (REVEILLE8)), whose defining member is TOC1 (TIMING OF CAB EXPRESSION 1,

also called PRR1). In addition to these “core components”, the GIGANTEA (GI) and the evening complex regulators (EC, formed by ELF3, ELF4 and LUX/NOX) play also a prevalent role in self-sustaining oscillation of these genes (Somers 2012).

The current model for the central oscillator implies multiple interconnected negative feedback loops that function at repression of a tri-component negative feedback ring comprised by CCA1/LHY, PRRs and EC, and where each of these components represses the next component in the ring (Pokhilko, Fernandez et al. 2012) (Figure 6a). Although this model is based on the ability of these regulators to repress each other’s expression, increasing evidence supports also an additional role of these proteins in translational regulation, on top of chromatin remodeling events and a critical role of alternative splicing in the regulation of some of these genes (Stratmann and Mas 2008; Nagel and Kay 2012; Troncoso-Ponce and Mas 2012). In a more simplified model (reviewed in (Nagel and Kay 2012)), the MYB transcription factors, CCA1 and LHY, which peak in the morning, repress expression of the EC, *GI* and *TOC1* genes, by directly binding to their promoters, hence indirectly activating *PRR9* and *PRR7* gene expression (Nagel and Kay 2012). The PRR9, -7, -5 repressors, in turn, bind sequentially to the CCA1/LHY promoters and repress expression of these genes during the day to midnight. Each of these PRR proteins, is active at a specific time of the day, PRR9 functioning at early daytimes, PRR7 from early day to midnight and PRR5 from noon to midnight (Nakamichi 2011). Sequential suppression of *CCA1/LHY* gene expression, relieves CCA1/LHY repression on *TOC1* and on the evening phased *GI* and EC genes, and activates the expression of these genes, with levels of these transcripts increasing during the day to peak at dusk (Nagel and Kay 2012). TOC1 accumulation during early night, in turn, is thought to repress *CCA1/LHY* expression and restrict accumulation of these factors to late night (Gendron, Pruneda-Paz et al. 2012; Huang, Perez-Garcia et al. 2012). Such temporal action of TOC1, however, needs to be further confirmed since recent findings point to an additional function of this protein during the day (Somers 2012).

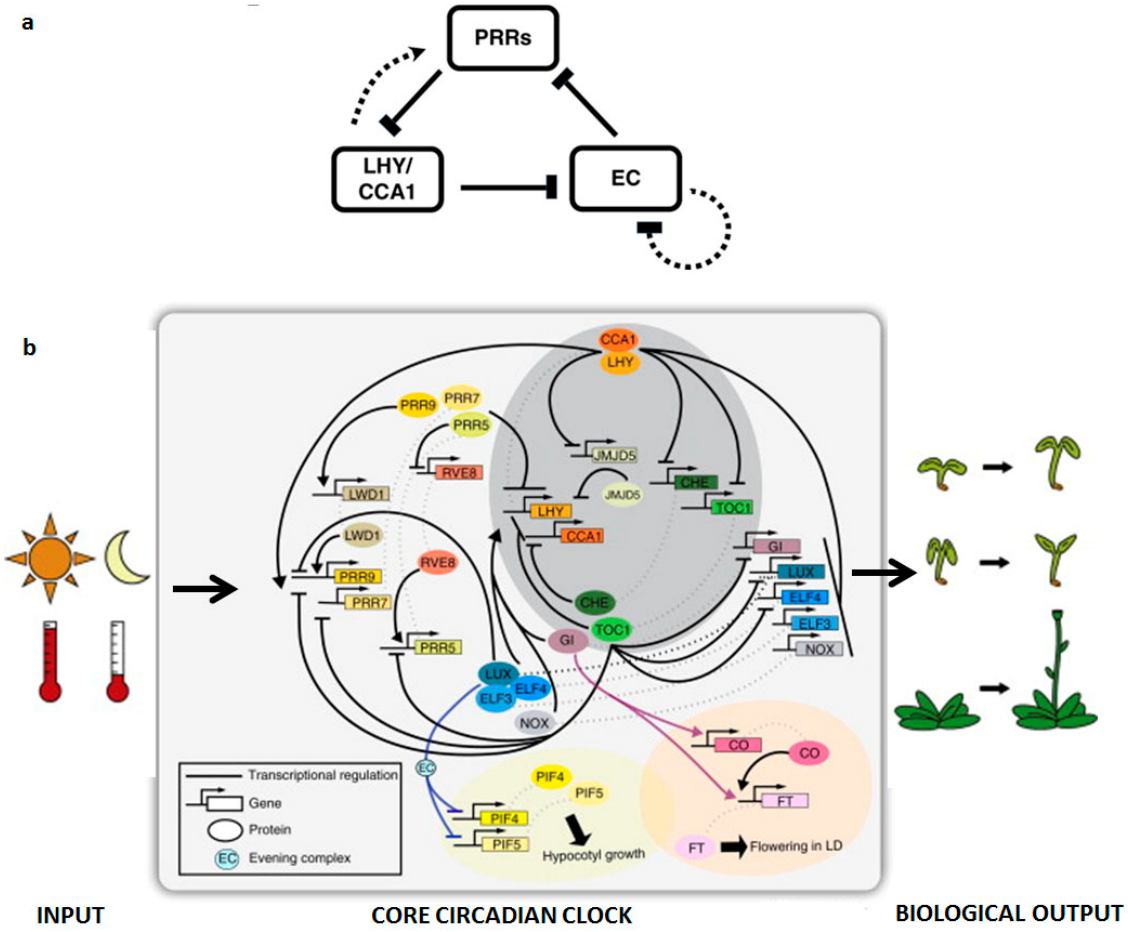

Figure 6. Simplified version of the clock. a) The central oscillator functions as a repressor chain. A tri-component (CCA1/LHY, PRRs and EC) negative feedback ring comprises the "core" of this oscillator. Each of these components represses the next component in the ring and indirectly activates the third of these components, by repressing its repressor. Figure adapted from (Pokhilko, Fernandez et al. 2012). b) Inputs such as light and temperature synchronize the central oscillator and these signals are transduced into different outputs that modulate photomorphogenic responses. In this simplified model, CCA1/LHY represses all evening phased genes, with exception of *PRR-9* and *-7*. PRRs, in return, repress *CCA1/LHY* expression. Repression of these repressors activates expression of the *EC* and *TOC1* genes. These repressors, in turn, inhibit *PRR*s expression, delay expression of *CCA1/LHY* to late night, and transduce information of the central oscillator to modulate photomorphogenic responses such as hypocotyl elongation, via EC repression of the *PIF4* and *PIF5* genes, or floral transition, by GI-mediated activation of the *CO* and *FT* genes. Figure adapted from (Nagel and Kay 2012).

TOC1, GI and EC, in addition, have been reported to positively regulate *CCA1/LHY* expression, through an unknown mechanism. This regulation may be indirect, since TOC1 and EC repress *PRR9*, *-7* and *-5* transcription (Nagel and Kay 2012), which

leads to the release of *CCA1/LHY* repression and to increased CCA1/LHY protein levels during late night and the morning (Kolmos, Herrero et al. 2011). GI, on the other hand, negatively modulates TOC1 protein levels, by stabilizing the blue light absorbing ZEITLUPE (ZTL) F-box protein, that directs TOC1 and PRR5 to degradation (Nakamichi 2011). Hence, enhanced degradation of TOC1 may further contribute to release the repression imposed by TOC1 on *CCA1/LHY* expression. This simplified model of regulation is completed by the negative feedback loop of the EC, that represses expression of all this complex components, with the exception of ELF3 (Figure 6b) (Nagel and Kay 2012)..

Resetting and gating the clock.

The central oscillator is in itself a self-sustaining mechanism that enables anticipating to the daily fluctuations in environmental conditions. However, as these conditions change, variations in light and temperature are utilized as main inputs to the clock to set the time of this oscillator. This external inputs, called “zeitgebers” (time-givers), reset the circadian clock at dawn, in a process called entrainment, which is essential to synchronize the clock with the local time conditions (Jones 2009). Light inputs perceived by the different families of photoreceptors are transduced to the clock via an intricate network of interactions that modulate transcription of different clock components, and also regulate some of these components at the protein level. Interestingly, photoreceptors are essential to set the phase of the clock but do not function as core components of this central oscillator (Yanovsky, Mazzella et al. 2000; Strasser, Sanchez-Lamas et al. 2010). CRY and PHY expression shows a rhythmic oscillation pattern under constant conditions, these genes therefore being also regulated as outputs of the clock (Toth, Kevei et al. 2001). Ability of light to reset the clock depends on the time of the day, since a pulse of light given before dawn advances the phase of the clock, yet the same pulse given after dusk delays the phase or is of no effect at noon time. Such variation in the sensitivity to external cues is known as “gating” of the clock and it defines a phase response curve or interval of the day in which the oscillator responds to environmental stimuli. The molecular mechanisms implicated in such repressive gating effect are poorly understood (Herrero-Soriano 2011), although notably, this response is lost in the *elf3* mutants, that show constitutive activation of the *CAB2* gene in constant light (McWatters, Bastow et al. 2000). Phase shifts induced by light pulses during the night, are also reduced in ELF3ox lines, indicating that ELF3 plays a key role in phototransduction to the oscillator, by negatively modulating responses to the light inputs (McWatters, Bastow et al. 2000;

Covington, Panda et al. 2001). This protein of unknown function was first identified because loss-of-function mutations in this gene cause early flowering, independently of day length, and lead to a characteristic pale leaf and elongated hypocotyl and petioles phenotype, usually associated with defective light perception (Zagotta, Hicks et al. 1996; Liu, Covington et al. 2001). These mutants were later shown to be impaired in the clock entrainment responses to temperature, and to red and blue light, and to be arrhythmic in constant light but not in darkness, hence confirming a prevalent role of the ELF3 protein in the light (Hicks, Millar et al. 1996; Covington, Panda et al. 2001; Thines and Harmon 2010). The *elf3* mutant actually shows an increased hypocotyl size in white, blue and red lights, but not in darkness (Zagotta, Hicks et al. 1996). In studies in which the acute response to light of the *CAB2* (*Chlorophyll A/B binding protein 2)* gene was measured, as a readout of clock function, it could be observed that the peak of ELF3 protein coincides with the minimal responsiveness to light of *CAB2* (McWatters, Bastow et al. 2000; Covington, Panda et al. 2001). Thus, ELF3 appears to play a central role in the process of gating, by desensitizing transduction of the input light signals to the oscillator (Carre 2002). The role of ELF3 in sustaining the oscillator, on the other hand, can be separated from its role in resetting and gating the clock, since a mutant allele (*elf3-12*) of this gene has been recently described, that although altered in clock function it still displays rhythmic oscillations with a shortened period in the light and does not show the developmental defects associated to the null *elf3* mutant (Kolmos, Herrero et al. 2011). Over-expression of PHYA and PHYB enhances the short period effect of this mutation, *elf3-12* seedlings being in addition impaired both in regulation of light-induced gene expression and in resetting of the oscillator. Thus, the early flowering and long hypocotyl phenotypes of *elf3* null mutants appear to result from an impaired function of the clock and not just from defects in the light input to the oscillator (Kolmos, Herrero et al. 2011).

Early Flowering 3 (ELF3).

The *ELF3* gene is rhythmically expressed and encodes a protein of 695 amino-acid with a nuclear localization. In seedlings subjected to diurnal conditions, the peak of expression of this gene coincides with 12 hours after the last dark-to-light transition, independently of short or long days. In free running conditions, this gene peaks in the middle of the subjective night (Covington, Panda et al. 2001; Hicks, Albertson et al. 2001; Liu, Covington et al. 2001). Protein levels also oscillate during the day, following the expression profile of the gene (Liu, Covington et al. 2001; Yu, Rubio et al. 2008;

Nusinow, Helfer et al. 2011), although final protein levels are controlled by the E3 ligase COP1, that destabilizes this protein (Yu, Rubio et al. 2008). ELF3 homologues have been identified in all plant species, including monocots and dicots. Alignment of the predicted proteins identified four conserved domains, unique to these proteins, that do not share similarity with other domains of known function (Liu, Covington et al. 2001) (Figure 7a). These proteins are particularly rich in serine, proline and glutamine/threonine regions (Carre 2002), a polyglutamine-repeat tract in the second half of the protein, that exhibits polymorphism between different *Arabidopsis* accessions, being shown to be associated with a quantitative trait loci (QTL) for differential response to shade avoidance of these accessions (Jimenez-Gomez, Wallace et al. 2010; Coluccio, Sanchez et al. 2011) and to drive adaptation to internal genetic environments (Undurraga, Press et al. 2012). Up to the date, the biochemical function of ELF3 protein is poorly understood albeit recent evidence indicates that this protein may function as a scaffold protein that mediates PHYB and COP1 interaction with different protein partners (Yu, Rubio et al. 2008; Nusinow, Helfer et al. 2011). Interacting partners have been identified, which bind the N-terminal (N-ELF3) and the central (M-ELF3) domains (Figure 7b). The N-ELF3 region binds PHYB and COP1 (Liu, Covington et al. 2001; Yu, Rubio et al. 2008), interaction of these proteins with the same domain hence suggesting a competitive function of these two factors in the regulation of ELF3 activity, although such antagonistic function has not been yet demonstrated. Noteworthy, the N-ELF3 domain is not essential for circadian clock function, although it has been hypothesized that it mediates light repression of ELF3 activity via interaction with PHYB (Herrero-Soriano 2011). Both the N-ELF3 and M-ELF3 domains were reported to be implicated in the recruitment of the GI-ELF3-COP1 complex, with ELF3 serving as an adaptor protein that couples GI to COP1. Formation of this ternary complex leads to GI and ELF3 ubiquitination and to targeted degradation of these proteins, hence modulating flowering time in short day conditions (Yu, Rubio et al. 2008). Regarding modulation of ELF3 protein stability, this protein is detected in the nucleus in the light while in darkness, it decays to undetectable levels, hence suggesting a possible role of COP1 in ELF3 destabilization in the absence of light (Liu, Covington et al. 2001; Yu, Rubio et al. 2008). The M-ELF3 domain, in turn, mediates ELF3 interaction with the clock protein ELF4, this interaction activating ELF3 function, possibly by increasing nuclear abundance of this protein (Herrero, Kolmos et al. 2012). Interaction with ELF4 also mediates assembly of the evening complex (EC), by subsequent binding of LUX ARRHYTHMO (LUX) that targets this complex to the

promoters of its regulated genes (Nusinow, Helfer et al. 2011). The C-terminal domain (C-ELF3) is required for ELF3 nuclear localization (Herrero, Kolmos et al. 2012) and this domain was recovered in a yeast two-hybrid screen in which ELF3 was used as bait, which suggests that this domain would be implicated in dimerization of the protein (Liu, Covington et al. 2001) (Figure 7b).

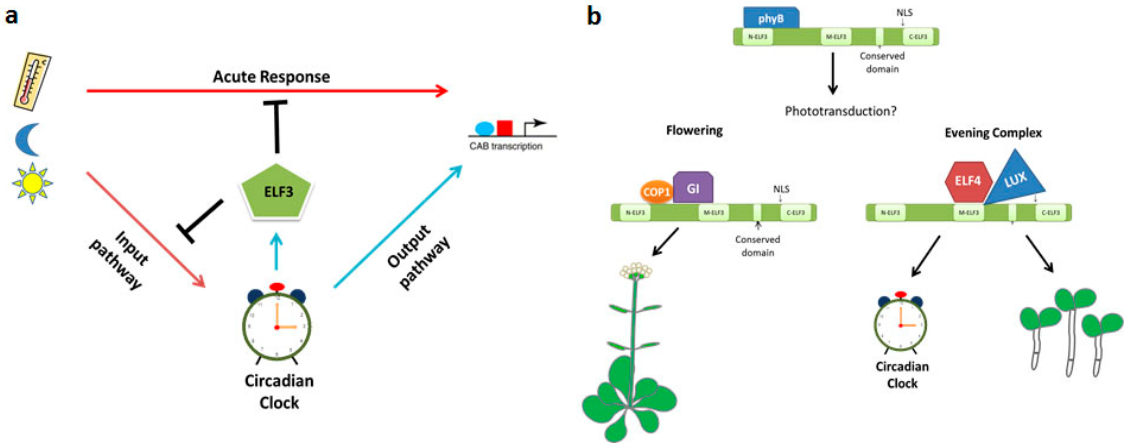

Figure 7. ELF3 is a clock component and modulates both gating and resetting of the clock. a) ELF3 is a key component of the phototransduction pathway to the clock and it also modulates clock sensitivity to the input signals (Thines and Harmon 2010). Figure adapted from (Carre 2002). b) The ELF3 protein. ELF3 has four conserved domains that do not share similitude with other proteins of known function (Liu, Covington et al. 2001). The N-terminal domain (N-ELF3) mediates interaction with COP1 and phyB (Liu, Covington et al. 2001; Yu, Rubio et al. 2008) while the central domain (M-ELF3) is implicated in GI and ELF4 interaction (Liu, Covington et al. 2001 (Nusinow, Helfer et al. 2011). The C-Terminal domain includes a putative nuclear localization signal (NLS) and mediates dimerization of the protein (Liu, Covington et al. 2001; Herrero, Kolmos et al. 2012). Recent evidence has shown that ELF3 functions as an adaptor or scaffold protein for GI and COP1 interaction, to regulate flowering time in short day photoperiods (Rubio et al. 2008). Formation of the ELF3-GI-COP1 ternary complex leads to GI and ELF3 degradation. This protein also recruits ELF4 and LUX/NOX to form the evening complex (EC), a negatively regulatory complex that represses transcription of the *PRR9* and *PIF4/PIF5* genes, to respective modulate the circadian clock and hypocotyl growth (Nusinow, Helfer et al. 2011; Nagel and Kay 2012).

Mutations in the ELF3 gene lead to phenotypic changes at the whole plant level, although tissue-specific regulatory functions of this protein have been also described. In a screening for mutations that suppress the closed stomata phenotype of *phot1phot2* mutants, for example, a novel *elf3* null mutant allele could be identified, which displays open-stomata, high H(+)-ATPase activity and increased FT expression in guard cells (Kinoshita, Ono et al. 2011). This observation uncovers a new cell-autonomous role for

FT and demonstrates that ELF3 and FT are involved in the blue light regulation of H(+)-ATPase in guard cells (Kinoshita, Ono et al. 2011). Interestingly, additional studies showed that the rhythmic modulation of water dynamics and aquaporin expression is also impaired in the *elf3* mutant (Takase, Ishikawa et al. 2011), hence opening a new field of research that connects the circadian clock with the diurnal regulation of stomatal function and water dynamics.

The ELF3 protein is in a way an atypical protein since regions in between its conserved domains are enriched in secondary structure-breaking residues, such as proline. This would suggest that ELF3 forms a linear unfolded structure that leaves exposed the different conserved domains, for interaction with a diverse array of proteins. All lines of evidence actually point to a function of this protein as a scaffold for complex association with different interacting partners, although more research will be required to confirm this biological function. At the time this work was started, it had been established that loss-of-function mutations in this gene lead to a phenotype associated with defective light perception, such as pale leaves and elongated hypocotyl and petioles, but the molecular mechanism underlying this phenotypic effects remained elusive until quite recently.

Circadian regulation of hypocotyl growth.

As seed germinates, rapid elongation of the hypocotyl towards the soil surface, to reach light, is a committed step ensuring seedling survival. This rapid elongation growth is controlled by several pathways that converge in the PIF4 and PIF5 proteins. In darkness, elevated levels of gibberellins promote the degradation of DELLAs, allowing PIFs to accumulate in a free form that is transcriptionally active. Phytochromes remain inactive in darkness, hence promoting hypocotyl elongation by stabilization of the PIF4 and PIF5 proteins (de Lucas, Daviere et al. 2008; Leivar and Quail 2011). Noteworthy, mutants affected in circadian clock function exhibit altered hypocotyl elongation phenotypes (Nozue and Maloof 2006), although how alterations in clock rhythmicity are linked to this photomorphogenic response has remained elusive. In hallmark studies in which hypocotyl growth was measured during the day (Nozue, Covington et al. 2007), it could be established that hypocotyl growth exhibits a rhythmic diurnal pattern that coincides with the end of the night period. These studies also showed that the clock modulates transcription of the *PIF4* and *PIF5* genes, these transcripts beginning to accumulate at the end of the night and peaking during the day, to be repressed at dusk (Nozue, Covington et al. 2007). In a complementary approach,

it was also shown that day length modulates *PIF4* and *PIF5* expression profiles and the levels of accumulation of these proteins, thus resulting in changes in the pattern of hypocotyl growth (Niwa, Yamashino et al. 2009). This observation let to postulate that long-day conditions would be insufficient to open the clock-gate for triggering *PIF4*/*PIF5* expression during the night, photoperiodic control of hypocotyl elongation thus being best explained by the accumulation these proteins at the end of the night in short days, due to coincidence between the internal (circadian rhythm) and external (photoperiod) time cues (Nozue, Covington et al. 2007; Niwa, Yamashino et al. 2009). The clock components regulating diurnal expression of the *PIF4* and *PIF5* genes were still unknown by the time that these results were published, but in a recent report it has been established that ELF3 interacts with the ELF4 and LUX/NOX proteins to form the so called evening complex (EC), a transcriptional repressor complex that binds the *PIF4* and *PIF5* promoters, to inhibit expression of these genes during early night (Nusinow, Helfer et al. 2011). Thus, it is now clear that PIFs are direct outputs of the clock. These transcriptional regulators in addition integrate light (PHYB) and GA (DELLAs) signals, with the endogenous clock, via its transcriptional repression by the evening complex (EC), which leads to promotion of hypocotyl elongation basically at the end of the night.

Regretfully, none of these models links the repressive activity of DELLAs with the observed diurnal pattern of hypocotyl growth. DELLAs were found to accumulate in the nucleus in the light, but to be destabilized in the dark, possibly due to increased bioactive GA synthesis during the night (Achard, Liao et al. 2007). In a recent effort to integrate GA signaling with the circadian clock, it could be shown that sensitivity to these hormones is gated via rhythmic expression of the GID1 receptors, which is controlled by the clock (Arana, Marin-de la Rosa et al. 2011). *GID1* gene expression was shown to peak at night, what coincides with the interval of the day in which DELLAs are destabilized. Expression of these receptors, in opposite, is low during the day, which is thought to contribute to increase stability of these repressors during daytime (Arana, Marin-de la Rosa et al. 2011). DELLAs were also shown to be required for the rhythmic growth of *Arabidopsis* seedlings, as the quadruple *rga-24 gait-6 rgl1 rgl2* mutant lacks rhythmicity in growth (Arana, Marin-de la Rosa et al. 2011). However, which components of the circadian clock modulate diurnal expression of the *GID1* genes or the exact mechanism by which DELLA proteins contribute to the rhythmic growth of seedlings, is still unknown.

Objectives of the work.

From all above discussed evidences, it is well established that PIFs play a central role in the control of seedling elongation. These transcription factors bind to G-box and E-box elements in the promoters of several genes encoding cell-wall remodeling enzymes, to activate their expression. Increased levels of expression of these genes lead to cell wall loosening, which is an essential step for cell elongation growth. Transcriptional activity of PIFs has been demonstrated to be highly regulated to restrict growth to the end of the night period and also to prevent excessive seedling elongation. Light is one of the major cues controlling PIFs activity, due it induces rapid degradation of these factors via direct interaction with the photoactivated PHYB red light receptor. These factors were shown in addition to be regulated in response to GA signaling, since they were shown to be bound by the DELLA repressors, to lead to an inactive complex unable to bind to DNA. However, it remained to be established how these signaling cues coordinate themselves to limit elongation growth to a small window of time at the end of the night period. Interestingly, in a two hybrid screen using the RGA protein as bait, it was observed that this DELLA repressor binds the protein ELF3, a poorly characterized protein with a described role in clock function and in resetting and gating the clock. This interaction looked very appealing, as it linked GA signaling with the endogenous clock. Complex formation with ELF3 may in fact modulate the stability or the repressive function of DELLAs and provide a molecular mechanism for circadian growth rhythmicity, since PIFs are accepted to accumulate in darkness. At the beginning of this work, we also uncovered that the ELF3 protein is able to directly interact with the PIF factors and thus may be implicated in regulating PIFs transcriptional activity. *ELF3* is actually transcribed late in the day, to peak at early night, thus a reasonable assumption was that the ELF3 protein represses PIFs transcriptional activity during the first half of the night. Therefore, the main objectives of this work were:

1. Confirm the ELF3-RGA and ELF3-PIF4 interaction *in vivo*.
2. Establish the biological implications of this interaction, by analyzing PIFs transcriptional activity and protein stability in *ELF3*ox lines and *elf3* mutant backgrounds.
3. Generate double *PIF4*ox *ELF3*ox and *PIF4*ox *elf3* lines, to analyze downstream regulated gene expression and hypocotyl growth of these plants.

4. Analyze the diurnal pattern of accumulation of the RGA repressor in *ELF3*ox and *elf3* mutant seedlings.
5. Test if ELF3 modulates GA biosynthesis/signaling, by analyzing the diurnal profiles of GA-related genes in ELF3 lines.
6. Establish a model of action of these regulators that contributes to understand rhythmicity in growth.

While carrying out this work, a new report on ELF3 function established that this protein binds ELF4 and the LUX/NOX factors, to form the evening complex (EC) that functions as one of the loops of the clock (Nusinow, Helfer et al. 2011). The GARP LUX factor mediates binding of this complex to the *PIF4* and *PIF5* promoters, to repress expression of these genes during early night. This introduced a new perspective to this work, but did not invalidate our original hypothesis, since we were still able to show that the ELF3 and PIF4 proteins interact *in vivo* and that over-expression of ELF3 restrains the excessive elongation phenotype caused by PIF4 over-expression, hence indicating that ELF3 represses PIF4 transcriptional activity. Thus, ELF3 appears to modulate PIF4 transcriptional activity at both the transcriptional and post-transcriptional levels, hence providing an additional layer of regulation to these important growth promoting factors.

Materials and Methods

Cloning procedures.

Most of the plasmid constructs were generated by the gateway system (pENTR™ Directional TOPO® Cloning kit and Gateway® LR Clonase™ II Enzyme Mix, Invitrogen), following the manufacturer recommendations. The PCR products amplified with specific primers to the desired genes were separated by gel electrophoresis and purified with the QIAGEN QIAquick® Gel extraction kit. The generated plasmid constructs were transformed into bacteria and analysed by miniprep extraction. Positive clones were further amplified and the plasmidic DNA purified with the QIAGEN QIAprep® Midi-Prep kit.

For conventional positional cloning, the genes of interest were amplified with specific primers that contained the adequate restriction sites and cloned into the pGEM®T-Easy vector (Promega) following the manufacturer instructions. The generated plasmids were then subjected to digestion with the desired restriction enzyme, dephosphorylated by digestion with the shrimp alkaline phosphatase (Roche), and ligated into the final plasmids previously digested with compatible enzymes, by incubation with the T4 *ligase* enzyme (Fermentas). The *E. coli DH5α* strain (Woodcock et al., 1989) was used for cloning and plasmid amplification. Cells were transformed by the heat-shock method (Sambrook et al., 1989), and grown in Luria-Bertani (LB) media, supplemented with the adequate antibiotics.

Plasmid constructs.

As entry plasmids for the gateway system the pENTR™/SD-TOPO® vector (Invitrogen) was used. Sequences inserted in this entry vector were mobilized to the final binary plasmids by the LR Clonase™. The pGWB1, pGWB6, pGWB14 and pGWB23 vectors (Nakagawa, Kurose et al. 2007) were used for fusion to GFP, and the MYC/ HA tags. pYL-CFP and pYL-YFP were a gift from Vicente Rubio. For luciferase carrying constructs, the pLuc-Trap3 vector (Calderon-Villalobos, Kuhnle et al. 2006) was used as destination plasmid.

For yeast two-hybrid studies, the pGADT7 and pGBKT7 vectors (Clontech) were used. Fragments were inserted into these vectors by conventional positional cloning as described in Table 3. Modified *Gateway* versions of these plasmids were generated by

insertion of the *Nde*I-*Xho*I Gateway *ccdB* cassette into the *Nde*I-*Sal*I sites of the vector multiple cloning site (MCS). Sequences inserted in the pENTR™-TOPO® vector were then mobilized into these vectors by incubation with the LR Clonase™, as before.

Table 1. pENTR™ constructs.

Construct	Plasmid	Selection marker	Observations	Cloning technique
pPIF4	pENTR™/SD-TOPO®	Kan+	Gift from de Lucas M.	TOPO
PIF4	pENTR™/SD-TOPO	Kan+	Gift from de Lucas M.	TOPO
pPIL1	pENTR™/SD-TOPO	Kan+	Gift from de Lucas M.	TOPO
ELF3	pENTR™/SD-TOPO	Kan+		TOPO
RGA	pENTR™/SD-TOPO	Kan+		TOPO
ELF4	pENTR™	Kan+	Gift from Jones M.A., UC Davis	
COP1	pENTR™/SD-TOPO	Kan+		TOPO
GAI	pENTR™/SD-TOPO			TOPO

Table 2. Binary constructs.

Construct	Plasmid	Selection marker	Observations	Cloning technique
pPIF4::LUC	pLucTrap3 (Calderon-Villalobos, Kuhnle et al. 2006)	Kan+	de Lucas M.	Gateway
pPIF4::PIF4-LUC	pLucTrap3 (Calderon-Villalobos, Kuhnle et al. 2006)	Kan+	de Lucas M.	Gateway

pPIL1::LUC	pLucTrap3 (Calderon-Villalobos, Kuhnle et al. 2006)	Kan+	de Lucas M.	Gateway
35S::ELF3-HA	pGWB14 (Nakagawa, Kurose et al. 2007)	Kan+		Gateway
35S::PIF4-HA	pGWB14 (Nakagawa, Kurose et al. 2007)	Kan+		Gateway
35S::ELF4-HA	pGWB14 (Nakagawa, Kurose et al. 2007)	Kan+		Gateway
35S::RGA-GST	pGWB23 (Nakagawa, Kurose et al. 2007)	Kan+		Gateway
35S::RGA-CFP	pYL-CFP	Spe^{+}		Gateway
35S::ELF3-YFP	pYL-YFP	Spe^{+}		Gateway
35S::ELF3-CFP	pYL-CFP	Spe^{+}		Gateway
35S::COP1-YFP	pYL-YFP	Spe^{+}		
*35S::*GFP-*GAI*	pPZP122/WB6	Spe^{+}	From Prat S.	Gateway
*35S::*MYC-*COP1*	pCAMBIA-1300-221	Spe^{+}	Gift from (Liu, Zhang et al. 2010)	

*35S::*GFP-MYC	pBA002	Spe^{+}	Gift from (Liu, Zhang et al. 2010)	
*c*YFC-*ELF3*	pYFC43 (Belda-Palazon, Ruiz et al. 2012)	Kan+		Gateway
*n*YFC-*RGA*	pYFN43 (Belda-Palazon, Ruiz et al. 2012)	Kan+		Gateway
nYFC-PIF4	pYFC43 (Belda-Palazon, Ruiz et al. 2012)	Kan+		Gateway
pBINAR	pBIN19	Kan+	(Hofgen 1990)	
P19	pBIN61	Kan+	(Voinnet, Pinto et al. 1999)	

Table 3. Yeast two hybrid constructs.

Construct	Selection marker	Observations	Cloning technique
pGBKT7 (GAL4 binding domain, bait)			
ELF3	Kan+		Gateway
N-ELF3	Kan+		Gateway
M-ELF3	Kan+		Gateway
C-ELF3	Kan+		Gateway
M5-GAI	Kan+	Described in (de Lucas, Daviere et al. 2008)	Positional cloning
M5-RGA	Kan+	*Bam*HI/*Pst*I	Positional cloning

M5-RGL1	Kan+	*Bam*HI/*Sal*I	Positional cloning
M5-RGL2	Kan+	*Bam*HI/*Pst*I	Positional cloning
M5-RGL3	Kan+	*Sal*I/*Pst*I	Positional cloning
RGA-F1	Kan+	Described in (de Lucas, Daviere et al. 2008)	
RGA-Relig	Kan+	Described in (de Lucas, Daviere et al. 2008)	
RGA-del1	Kan+	Described in (de Lucas, Daviere et al. 2008)	
RGA-del2	Kan+	Described in (de Lucas, Daviere et al. 2008)	
pGADT7 (GAL4 activation domain, prey)			
PIF4	Cb^+	Described in (de Lucas, Daviere et al. 2008)	
PIF4-del1	Cb^+	Described in (de Lucas, Daviere et al. 2008)	
PIF4-del2	Cb^+	Described in (de Lucas, Daviere et al. 2008)	
PIF4-del3	Cb^+	Described in (de Lucas, Daviere et al. 2008)	
PIF4-del4	Cb^+	Described in (de Lucas, Daviere et al. 2008)	
SPT	Cb^+	Described in (Gallego-Bartolome, Minguet et al. 2010)	
PIF1	Cb^+	Described in (Gallego-Bartolome, Minguet et al. 2010)	
HFR1	Cb^+	Described in (Gallego-Bartolome, Minguet et al. 2010)	
ELF3	Cb^+		Gateway

N-ELF3	Cb^+		Gateway
M-ELF3	Cb^+		Gateway
C-ELF3	Cb^+		Gateway
Large	Cb^+	BamHI	Positional cloning
Small	Cb^+	BamHI/EcoRI	Positional cloning

Plant material and growth conditions.

Seeds where sterilized in 70% ethanol 0.1% Tween-20 for 15 minutes with agitation, and washed twice with 100% ethanol for 2 minutes with agitation. After removing the ethanol, they were air dried for 2 hours and plated in the respective medium of germination. Seeds were then stratified for 2-3 days in darkness at 4ºC and plates transferred to the growth chambers for growth.

For short day and long day experiments in house chambers set at 22ºC were used. Light at 70 µmol $m^{-2}s^{-1}$ intensity was provided by incandescent tubes (PHILIPS TLD-D 30W/33-640 and SYLVANIA GRO-LUX F30W/GRO). Monochromatic light experiments were done in a Percival trichromatic light chamber (Mod. E-30LEDL3) adjusted at 22ºC. For red light studies, LEDs were set at 35 µmol $m^{-2}s^{-1}$ PAR, whereas for far-red light studies the intensity of LEDs was set to 15 µmol $m^{-2}s^{-1}$ PAR. Dark experiments were performed in the same chamber, with all monochromatic lights switched off.

For all experiments plant material was carefully collected with forceps for microscope observation or directly frozen in liquid N_2, for protein or RNA extraction. A green safe light was used to collect the material in darkness. For MG132 (Calbiochem), cycloheximide (SIGMA), GA_3 (Duchefa) and estradiol (SIGMA) treatments, plants where transferred to liquid 0.5x MS medium containing these substances, and incubated in parallel in 0.5x MS medium as a mock control. Seedlings were dried with paper to remove the excess of liquid and frozen in liquid N_2 for subsequent protein or RNA extraction.

0.5x MS: MES 0.5g, MS salts 2.2g, sucrose 10g, plant agar (Duchefa) 5.5g/ L
Adjust to pH 5.8 with KOH.

Hypocotyl length measurements.

To determine seedling's hypocotyl length, seeds were plated in solid 0.5x MS medium and grown vertically. For vertical plates, 5.8 g plant agar was used per litre, to increase the texture strength of the media. Plates were photographed at different days after sowing (growth kinetics) or at day 5 of growth. Images were analysed with the *ImageJ* image processing program (rsbweb.nih.gov/ij/), by measuring the length of the hypocotyls from the apical meristem to the point of transition to the root. The average and standard deviations of at least 10 plants were plotted for each experiment.

Mutant seedlings and transgenic lines.

All *Arabidopsis* mutants and transgenic lines were in the *Columbia* (*Col-0*) ecotype, except for *sly1-10 (de Lucas, Daviere et al. 2008)* and the *tetra (de Lucas, Daviere et al. 2008)* mutants that are in the *Lansdberg* erecta (*Ler*) ecotype.

Table 4. Mutant alleles and *Col-0* transgenic lines.

Stable lines	Ref	Observations	TAG
pif4-101	(de Lucas, Daviere et al. 2008)		
pif5-1	(de Lucas, Daviere et al. 2008)		
pif4-101pif5-1	(de Lucas, Daviere et al. 2008)	*pif4pif5*	
phyB pif4-101pif5-1	(de Lucas, Daviere et al. 2008)	*phyBpif4pif5*	
phyB	(de Lucas, Daviere et al. 2008)		
*35S::*PIF4-HA	(de Lucas, Daviere et al. 2008)		HA
*pPIF4::*PIF4-HA		Kan+ resistance. Gift from Stella Bernardo	HA

elf3-8	(Yu, Rubio et al. 2008)		
elf3-8cop1-4	(Yu, Rubio et al. 2008)		
cop1-4	(Yu, Rubio et al. 2008)		
cop1-6	(Yu, Rubio et al. 2008)		
35S::ELF3cop1-4	(Yu, Rubio et al. 2008)		
35S::ELF3	(Yu, Rubio et al. 2008)	*gl1 (GLABRA1)* background	
PIF4ox	(Khanna, Huq et al. 2004)	*PIF4* over-expresser under its native promoter.	
PHYBox			
COP1ox			
elf3-8 pif4-101	This study	Crossed	
elf3-8 pif5-1	This study	Crossed	
elf3-8 pif4-101pif5-1	This study	Crossed	
elf3-8 PIF4ox	This study	*PIF4* under its native promoter	
35S::ELF3 PIF4ox	This study	*PIF4* under its native promoter	
*35S::ELF3 35S::*PIF4:HA	This study	Crossed	HA

Table 5. Luciferase reporter transgenic lines.

Stable lines	Reference	Observations
pPIL1:::LUC	(Li, Ljung et al. 2012)	*Kan*+ resistance
elf3-8 pPIL1::LUC		Crossed, selected by phenotype and *Kan*+ resistance
35S::ELF3 pPIL1::LUC		Crossed, selected by phenotype and *Kan*+ resistance
pPIF4::LUC	This study	*Kan*+ resistance
pPIF4::PIF4-LUC	This study	*Kan*+ resistance
elf3-8 pPIF4::LUC		Crossed, selected by phenotype and *Kan*+ resistance
elf3-8 pPIF4::PIF4-LUC		Crossed, selected by phenotype and *Kan*+ resistance
35S::ELF3 pPIF4::LUC		Crossed, selected by phenotype and *Kan*+ resistance
35S::ELF3 pPIF4::PIF4:LUC		Crossed, selected by phenotype and *Kan*+ resistance

Table 6. Estradiol inducible lines.

(http://bioinfogp.cnb.csic.es/transplanta_dev/)

Stable lines	Reference	Observations
*XVE:*GFP-GUS	TRANSPLANTA	Named: *GUS*
XVE:PIF4	TRANSPLANTA	Named: *H59*

Table 7. GFP transgenic lines.

Stable lines	Ref.	Observations
*pRGA::*GFP-RGA	(Silverstone, Jung et al. 2001)	*Kan*+ resistance. *Col-0* background. NASC ID: N16360
*phyB pRGA::*GFP-RGA		Crossed, selected by phenotype and *Kan*+ resistance
*PHYBox pRGA::*GFP-RGA		Crossed, selected by phenotype and *Kan*+ resistance
*cop1-6 pRGA::*GFP-RGA		Crossed, selected by phenotype and *Kan*+ resistance
*COP1ox pRGA::*GFP-RGA		Crossed, selected by phenotype and *Kan*+ resistance
*elf3-8 pRGA::*GFP-RGA		Crossed, selected by phenotype and *Kan*+ resistance

To genotype lines carrying the *elf3-8* mutation, a 200 bp PCR fragment that includes this mutation was amplified by using primers ELF3_R and *dF(DdeI)*. The PCR product was digested with *Dde*I that cuts the WT *ELF3* allele giving rise to two bands, of 178bp and 23 bp. Primers ELF3_F and *dR(ScaI)* were also used to amplify a 190bp fragment of the mutant allele. This PCR fragment was digested with *Sca*I that cuts the *elf3-8* mutant allele to give two bands of 160 bp and 30 bp. The digestion products were analyzed in 5% agarose gels.

Transient expression studies.

For agroinfiltration of *Nicotiana benthamiana* leaves the *Agrobacterium tumefaciens* C58C1 strain was used. Plasmid constructs were transformed by heat shock into this strain according to (Endo, Nakamura et al. 2005). The 4-5 leaf *Nicotiana benthamiana* plants used for infiltration was provided by the CNB greenhouse service. *Agrobacterium* cells transformed with the different constructs were grown overnight and used for infiltration of the abaxial side of *N. benthamiana* leaves according to (Liu, Zhang et al. 2010). The same infiltration method was used for BiFC, luciferase assays and co-immnuprecipitation studies. After 3 days of infiltration, leaves were used for confocal microscope observation of the GFP activity, discs of the leaves were taken for

the luciferase assays, or several leaves were collected and frozen in liquid N_2 for subsequent protein extraction.

BiFC (Bi-molecular Fluorescent Complementation).

The plasmids for the complementation of YFP, pYFN43 and pYFC43, described in (Belda-Palazon, Ruiz et al. 2012) were used for BiFC. Constructs carrying PIF4, ELF3, COP1 or RGA were generated by the gateway system using their correspondent pENTRY™ construct. To test the interaction, the transient expression with the paired complementary constructs and their respective controls were agroinfiltrated in *N. benthamiana* leaves. A solution with 50μM MG132 treatment was infiltrated according to (Liu, Zhang et al. 2010) 12 hours before the analysis by confocal microscopy for YFP activity. Positive nuclei were photographed.

Luciferase activity assays.

For studies of *luciferase* activity in whole *Arabidopsis* seedlings, seeds were plated on solid 0.5x MS media. After 3 days of germination, seedlings were carefully transferred to 96 well microplates (Corning Inc., COSTAR 3362) filled with 175 μL of solid 0.5x MS medium and 35 μL of the 1x D-Luciferin substrate (SIGMA, product #L9504) per well. Microplates were then sealed with a transparent optical adhesive film (Kisker GbR, PCR-folie ultra clear RT-PCR, G060/UC-RT) and each well was perforated twice with a 0.3 mm needle to allow gas exchange. Seedlings were let acclimate for 12 hours, before measuring Luciferase activity. Levels of Luciferase activity per plantlet were measured every hour, for a total of 3-5 days, using the LB 960 Microplate Luminometer (Berthold). Registered values represent the average of counts per second in each well, during two successive seconds, with at least 12 plants per line being used in these studies. Average values of these 12 replicates were plotted as an estimate of the Luciferase activity levels in these plants. The Luminometer was housed in a growth chamber to maintain the plants under controlled growth conditions, and to be able to subject them to SD/LD photoperiods or to continuous light treatments. This machine was equipped with robotics software programmed to leave the plates out between measurements, to minimize interference with diurnal conditions, and was operated through the Windows® PC MikroWin 2000 software, that also serves as a data evaluation tool.

For trans-activation assays, leaves of *N. benthamiana* plants were co-infiltrated with

the *Agrobacterium* strains bearing the desired constructs and two days after agroinfiltration, 1cm Θ discs were collected from the leaves with the aid of a cylindrical borer and carefully transferred, the abaxial side upwards, to 96 well microplates filled with 175 µl liquid 0.5x MS and 35 µl of the 1x D-Luciferin substrate. One disc was used per well and at least 12 disc replicates per sample. Plates were sealed with a transparent optical adhesive film and measured as indicated above.

D-Luciferin substrate (SIGMA, #L9504): A 500x stock solution was prepared by dissolving 10 mg of D-luciferin in 1 ml of DMSO, and aliquots of 8 µl were stored at -80ºC. Each of these aliquots was diluted in 4 ml sterile water to prepare a 0.02 mg/ml D-luciferin working solution and 35 µl used per well.

Protein analysis and co-immunoprecipitation studies.

For protein analysis, the material frozen in liquid N_2 and stored at -80ºC was grounded to powder with the aid of a mortar or an electric stirrer (IKA, 2572200) pre-cooled with liquid N_2 and extracted with protein extraction buffer 1. Extracts were always kept on ice and clarified by centrifugation at 13000 rpm during 15 min at 4ºC. Supernatants were carefully collected into a new tube and centrifuged again for 10 min at 13000 rpm to remove all plant debris. This second supernatant was transferred to a new tube and the protein content was quantified by the Bradford protein assay method (Bio-Rad). Equal amounts of protein were mixed in a 1:1 ratio with TMx2 buffer and separated in 8% SDS-PAGE gels. Gels were transferred to a nitrocellulose membrane using a semidry Transfer blot system and the membranes stained with red Ponceau to test that the protein transfer was good. They were then saturated with 10% non-fat milk and used for immunodetection with the appropriate antibodies. Most of the membranes where cut below 42 kDa for incubation with the Rpt5 antibody, used as loading control. For *N. benthamiana* protein extracts the concentration of β-Mercaptoethanol in the extraction buffer was increased to 10 µM, to avoid oxidation of the proteins.

For co-immunoprecipitation experiments, the infiltrated *N. benthamiana* leaf material was extracted with protein extraction buffer 1, with exception of the RGA-ELF3 interaction studies, in which NaCl concentration in the extraction buffer was decreased to 100 mM. For co-inmunoprecipitation studies in *Arabidopsis* the frozen material was extracted with protein extraction buffer 2. Briefly, 1-2 grams of frozen material was grounded in a pre-cooled mortar and extracted with 3 ml of buffer. The homogenate

was clarified twice by centrifugation at 13000 rpm during 15 min, and the recovered supernatant transferred to a new tube. 100 µl of the protein extract were separated in a new tube to be used as the input fraction in subsequent analyses, and the rest was incubated with 50 µl of µMACS™ anti-tag MicroBeads (Miltenyi Biotec , anti-HA #130-091-122 or anti-GFP #130-091-125, depending on the tag attached to the protein of interest). Beads were incubated for 3 hours with rotation at 4ºC, and afterwards loaded into a 20µ MACS® Separation Column (Miltenyi Biotec , #130-042-701) placed in the µMACS separator (Miltenyi Biotec , # 130-042-602) and previously equilibrated with 200 µl of extraction buffer. 200 µl of the column eluate were collected as the unbound fraction for further analyses. The µColumn was then washed 5 times with 500 µl of extraction buffer, and 15 µl (or empty volume of the column) of TMx2 buffer with 10% of β-mercaptoethanol heated at 95ºC was added. The column was incubated for 5 min in this buffer, and 85 µl more of TMx2 buffer with 10% β-mercaptoethanol, heated at 95ºC were added to the column to collect the eluate that corresponds to the co-immunoprecipitated fraction. For western blot analysis, 40 µl of the input and unbound fractions, along with the 15 µl of empty volume and 20-60 µl of the eluted fraction were loaded into a 8% SDS-PAGE gels and transferred to a nitrocellulose membrane for immunodetection with the appropriate antibodies.

Protein extraction buffer 1: Tris-HCl 50 mM pH 8, NaCl 150 mM, (100 mM for ELF3-RGA interaction in *Nicotiana),* Triton X-100 1%, PMSF 100 µM (SIGMA, P7626), β-mercaptoethanol 5 µM for *Arabidopsis and* 10 µM for *Nicotiana* (MERCK), MG132 10 µM (VWR Calbiochem, # 474790), + protease inhibitors (Aprotinin 10µM (SIGMA, A1153), E-64 10µM (SIGMA, E3132), Leupeptin 10µM (SIGMA, L2023), Pepstatin 1µM (SIGMA, P5318)).

Protein extraction buffer 2: Tris-HCl 50 mM pH 8, NaCl 75mM, Nonidet 0.5% (Igepal CA630), Deoxycholate 0.05% (SIGMA, D4297), PMSF 100 µM (SIGMA, P7626), β-mercaptoethanol 5µM, MG132 20µM + protease inhibitors.

TMx2: Tris-HCl 0.125M pH 7.4, Glycerol 20%, SDS 4%, Bromophenol blue 0.04%.

Antibodies: anti-MYC, BD Pharmigen, No. 551102, Purified Mouse Anti-Human c-MYC IgG. Dilution 1:1000; **anti-RPT5**, BIOMOL, PW8375-0025, Rabbit anti-Rpt5 (S6a). Dilution 1:10000; **anti-GFP**, ROCHE, No. 11814460001, anti-Green Fluorescent Protein mouse IgG. Dilution 1:1000; **anti-HA**, ROCHE, No. 12013819001, Horseradish

Peroxidase (HRP) conjugated rat anti-HA monoclonal antibody. Dilution 1:1000; **anti-GST**, GE Healthcare, RPN1236, Horseradish Peroxidase (HRP) conjugated goat anti-GST polyclonal antibody. Dilution 1:1000; **anti-GFP**, MACS, No. 130091833, Horseradish Peroxidase (HRP) conjugated mouse anti-GFP monoclonal antibody. Dilution 1:1000; **anti-ELF3**, described in (Nusinow, Helfer et al. 2011). Rabbit IgG. Dilution 1:250; **anti-RGA**, described in (Willige, Ghosh et al. 2007). Mouse IgG. Dilution 1:1000; **anti-rabbit IgG**, GE Healthcare, NA9340. Dilution 1:20000; **anti-mouse IgG**, GE Healthcare, Code: NA931. Dilution 1:10000.

Western detection: For antibody detection, the membranes were incubated with the Supersignal® west pico maximum sensitivity substrate, PIERCE, # 34080, or the Supersignal® west femto maximum sensitivity substrate, PIERCE, # 34095, and exposed to a film. The choice of either of these substrates depended on the intensity of the signal (for a weaker signal the femto substrate was used).

RNA extraction and qPCR.

For RNA analysis, the frozen material was grounded in an eppendorf tube with the aid of a pre-cooled electric stirrer (IKA, 2572200) and extracted with 300 µl of buffer Z6. One volume of phenol-chloroform was added to the extract and mixed thoroughly with the vortex. The mix was then centrifuged at 13000 rpm for 30 minutes at 4ºC and the upper aqueous fraction carefully collected into a new tube. RNA was purified from this fraction with the High Pure RNA Isolation Kit (ROCHE, Ref. 11828665001), following the manufacturer instructions. One volume of the Lysis/Binding Buffer provided in the kit was added to the upper fraction recovered after centrifugation, for binding to the columns. The RNA obtained after cleaning with the kit was quantified in a NANODROP. For cDNA synthesis, 2 µg of total RNA was used for transcription with the Transcriptor First strand cDNA synthesis kit (ROCHE, 04379012001). The cDNA reaction was diluted 1:10 in water and used for qPCR analysis with primers specific to the desired genes. The FastStart Universal SYBR Green Master mix (ROCHE # 04913850001) was used for amplification, with the *PP2A* gene used as internal control. qPCR reactions were run in a 7500 Real-Time PCR system (Applied Biosystems, # 4351105) and the amplification data analyzed by the 7500 software v2.0.

Z6 buffer: Guanidine-HCl 8M, MES (pH 7.0) 20mM, EDTA 20 mM and 10% β-mercaptoethanol.

Yeast two-hybrid assays.

Yeast two hybrid experiments were done with the Matchmaker GAL4 Two-Hybrid System (Clontech, Cat No. K1604-1, K1605-1, 630303), using the *AH109* yeast strain (Clontech) for auxotrophy selection. Co-transformation of the GAL4-BD and GAL4-AD constructs was performed according to the small-scale LiAc transformation procedure described in the Yeast Protocols Handbook (Clontech, PT3024-1). Cells were selected in SD-LT medium for the presence of both pGADT7 and PGBKT7 plasmids, and overnight cell cultures grown in this selection media plated in the auxotrophic SD-LTH and SD-LTHA media to test for interaction. Yeast growth and handling was according to the Yeast Protocols Handbook.

SD-LT: SD Minimal Agar Base (Clontech, No. 630412) supplemented with the –Leu/–Trp DO Supplement (Clontech, No. 630412).

SD-LTH: SD Minimal Agar Base supplemented with the –Leu/–Trp/-His/-Ade DO Supplement (Clontech, No. 630412) and 0.003% adenine.

SD-LTHA: SD Minimal Agar Base supplemented with the –Leu/–Trp/-His/-Ade DO Supplement (Clontech, No. 630428).

Confocal microscopy.

For confocal microscope visualization of agroinfiltrated *N. benthamiana* leaves, 2 cm^2 sections from the leaves were carefully removed and mounted in water on a microscope slide with the abaxial side facing upward. Excess water was added before the slide cover, to avoid trapping any air bubbles. Fluorescence was detected with a LEICA TCS SP5 confocal microscope (Leica Microsystems) using the excitation Beam Splitter FW (TD 488/561/633) and an objective HCX PL APO lambda blue 63.0x1.20 WATER UV. For GFP (YFP) detection the emission bandwidth was set to 508nm - 573nm and for chlorophyll detection to 652nm - 751nm.

For GFP detection in *Arabidopsis* seedlings, 4-5 day old seedlings were used. Plantlets were carefully removed from the medium with a forceps and incubated for 30 seconds in 1x Propidium Iodide solution. They were then washed in sterile water, and mounted in 10% glycerol in a microscope slide, with special care not to trap air bubbles. GFP fluorescence was detected in a Zeiss Axiovert 200 microscope, using the Radiance 2100 Laser Scanning System (Bio-Rad) in combination with the 560 DCLPXR beam

splitter and HQ515/30 emission filter. Chlorophyll was filtered with HQ 660LP and visualized with an Argon laser (λ=488). Images were processed with the LaserSharp v5.0 (Bio-Rad) and LaserPix v.4 (Bio-Rad) software.

The DIURNAL database.

DIURNAL (Mockler, Michael et al. 2007) (http://diurnal.mocklerlab.org/) is a free access database platform where genome-wide gene expression results for different plant species and diurnal conditions are stored. This web-tool allows easily querying the expression profiles of particular genes or array of probes, hence providing a good estimation of the phase of peak expression of these particular genes, in plants grown under different conditions, as listed in Table 8.

Table 8. Experimental conditions used for circadian gene expression studies in the DIURNAL database.

Name	Age	Acc	Media	Tissue	Light	Light	Temp	Lab
Diurnal conditions								
LLHC	7	*Col-0*	agar no suc	seedlings	100μE	LL	12h22ºC/ 12h12ºC	Chory, Michael
LDHC	7	*Col-0*	agar no suc	seedlings	100μE	12L/12D	12h22ºC/ 12h12ºC	Chory, Michael
LDHH-Stitt	35	*Col-0*	Soil	Leaves	130μE	12L/12D	22ºC	(Blasing, Gibon et al. 2005)
LDHH-Smith	29	*Col-0*	Soil	Leaves	180μE	12L/12D	20ºC	(Smith, Fulton et al. 2004)
Short Day	7	Ler	agar 3% suc	seedlings	180μE	8L/16D	22ºC	Kay, Yanovsky
Long Day	7	Ler	agar 3% suc	seedlings	90μE	16L/8D	22ºC	Kay, Yanovsky
Free running conditions								
LL (LLHC)	9	*Col-0*	agar no suc	seedlings	100μE	LL	22ºC	Chory, Michael
LL (LDHC)	0	*Col-0*	agar no suc	seedlings	100μE	LL	22ºC	Chory, Michael
LL12 (LDHH)	7	*Col-0*	agar 3% suc	seedlings	100μE	LL	22ºC	Kay, Harmer
LL23 (LDHH)	8	*Col-0*	agar 3% suc	seedlings	60μE	LL	22ºC	(Edwards, Anderson et al. 2006)
DD (DDHH)	8	*Col-0*	agar 3% suc	seedlings	0μE	DD	22ºC	Kay, Hazen
Added on 4/29/08								
COL SD	7	*Col-0*	agar no suc	seedlings	100μE	8L/16D	22ºC	Chory, Michael
COL LDHH	7	*Col-0*	agar 3% suc	seedlings	120μE	12L/12D	22ºC	Kay, Hazen
lux-2 LDHH	7	*lux-2*	agar 3% suc	seedlings	120μE	12L/12D	22ºC	Kay, Hazen

phyB-9 SD	7	*phyB-9*	Agar no suc	seedlings	100µE	8L/16D	22ºC	Chory, Michael

SCOPE.

SCOPE (Suite for Computational identification of Promoter Elements, http://genie.dartmouth.edu/scope/), is a web-based tool for the identification of *cis*-regulatory elements in a sequence or group of sequences. This tool was used to search for described motifs in the plus and minus strands of the 2500 bp upstream promoter region of *RGA.*

Table 9. List of primers.

	Name	Sequence	Observations
	Genotyping		
1	*dF(DdeI)*	AAACCTCTTCAACTGTGTAATACTCA	ELF3
2	*ELF3_R*	GTCAGTCTTCTCCGAGTCACC	ELF3
3	*ELF3_F*	CTCTGTTTCTCATTACAGTC	*elf3-8*
4	*dR(ScaI)*	ATCCGGTGATGCAGCAATAAGTTTTAGTAC	*elf3-8*
	Cloning		
5	*ELF3_Fb*	CACCATGAAGAGAGGGAAAGATGAGG	Gateway
6	*ELF3_Rb*	AGGCTTAGAGGAGTCATAGCG	No stop
7	*ELF3_L2*	CGGGATCCTGATGAAGAGAGGGAAAGATGAGG	BamHI restriction site N-ELF3 for Y2H
8	*ELF3_R2*	CGGGATCCTTGCCAAGTGAGATTCAGCTCC	BamHI restriction site N-ELF3 for Y2H
9	*ELF3_L3*	CGGGATCCTGGCAACGGAAAATCATTCACAAG	BamHI restriction site M-ELF3 for Y2H
10	*ELF3_R3*	CGGGATCCTGTAGTTGGATTGTTGATGATG	BamHI restriction site M-ELF3 for Y2H

11	*ELF3_L4*	CGGATCCATACATGCTTTTGCAAACAAACC		BamHI restriction site C-ELF3 for Y2H
12	*ELF3_R*	CGGGATCCTTAAGGCTTAGAGGAGTCATAG		BamHI restriction site C-ELF3 for Y2H
13	*M5GAI_F*	GGATCCTGGTTGACTCGCAGGAGAACG		BamHI
14	*M5GAI_R*	CTGCAGTCAACGTTTCATGACGCTCAA		PstI
15	*M5RGA_F*	GGATCCTGGTTGACTCGCAAGAGAACG		BamHI
16	*M5RGA_R*	CTGCAGGCTAAACCGGACGAACCAAAC		PstI
17	*M5RGL1_F*	GGATCCCTACGCGCTCTGTGG		BamHI
18	*M5RGL1_R*	GCGTCGACTTTATTCCACACGATTGAT		SalI
19	*M5RGL2_F*	GGATCCGACTCGGATCGTGGTGCGAAT		BamHI
20	*M5RGL2_R*	CTGCAGGACCCGATCGGACCCTTCCGC		PstI
21	*M5RGL3_F*	GCGTCGACCCGTGGTGCTTATCGAGG		SalI
22	*M5RGL3_R*	CTGCAGTCTACCGCCGCAACTC		PstI
23	*pPIL1 _F*	CACCGTGGTCTCATGAGTTCAGGCG		Gateway
24	*pPIL1 _R*	CTTCCGTTGAAGTAAACTGAACAAAGC		
25	*RGA-YFP-F*	CACCATGAAGAGAGATCATCAC		Gateway
26	*RGA-YFP-R*	GTACGCCGCCGTCGAGAG		No stop
27	*COP1_F*	CACCACAAAATGGAAGAGATTTC		Gateway
28	*COP1_R*	CGCAGCGAGTACCAGAACTT		No stop
	qPCR			
	PP2A 5'	TAACGTGGCCAAAATGATGC	Forward	(Czechowski, Stitt et al. 2005)

	PP2A 3'	GTTCTCCACAACCGCTTGGT	Reverse	(Czechowski, Stitt et al. 2005)
	AtGA3ox1	CCATTCACCTCCCACACTCT′	Forward	(Mitchum, Yamaguchi et al. 2006)
	AtGA3ox1	GCCAGTGATGGTGAAACCTT	Reverse	(Mitchum, Yamaguchi et al. 2006)
	GA2ox1-F	TGAGGACGAGAGGTTGTACGA	Forward	(Rieu, Eriksson et al. 2008)
	GA2ox1-R	TCCTTTCGAATTGTTGAAGCC	Reverse	(Genoud, Schweizer et al. 2008)
	PIL1_F	TATGCGGACCCTTCAACTTC	Forward	
	PIL1_R	GGCAACATCGTAGGTGGTCT	Reverse	
	ELF3_F	GGAAAGCCATTGCCAATCAA	Forward	(Wenden, Kozma-Bognar et al. 2011)
	ELF3_R	ATCCGGTGATGCAGCAATAAGT	Reverse	(Wenden, Kozma-Bognar et al. 2011)
	ATHB2_F	CATGAGCCCACCCACTACTT	Forward	
	ATHB2_R	ACCTAGGACGAAGAGCGTCA	Reverse	
	PIF4_F	TTAATCCGAACGCAAGTTCC	Forward	
	PIF4_R	CTCCTTCGGTTTGATCCTGA	Reverse	
	XTR7_F	TGGCGACTGTTCTTCTTGTG	Forward	
	XTR7_R	TCCTCCGTTGAAGATTTTGC	Reverse	
	GA20ox1_F	GATCCATCCTCCACTTTAGA	Forward	(Genoud, Schweizer et al. 2008)

	GA20ox1_R	GTGTATTCATGAGCGTCTGA	Reverse	(Rieu, Eriksson et al. 2008)
	GID1a_F	GTGACGGTTAGAGACCGCGA	Forward	(Arana, Marin-de la Rosa et al. 2011)
	GID1a_R	TCCCTCGGGTAAAAACGCTT	Reverse	(Arana, Marin-de la Rosa et al. 2011)
	IAA19_F	GAGCTGAGATTGGGGCTTC	Forward	
	IAA19_R	CCGACGACGTCATATTCATCT	Reverse	
	RGA_F	AATAGTGGCCAAGGTTATCGT	Forward	
	RGA_R	AGTGTGCCAACCCAACATC	Reverse	

CHAPTER 1: ELF3-PIF4 interaction uncovers a novel protein cascade loop involved in gating light-regulated responses of the clock in *Arabidopsis.*

Introduction.

In studies directed to elucidate the molecular mechanism of repression of DELLAs, several putative interacting proteins of the *Arabidopsis* GAI protein were identified in yeast two-hybrid screens with this bait protein. Functional characterization of one of these interactions, allowed generating a model of action for these negative regulators and has provided important insights concerning how plants integrate both light and GA signals to orchestrate growth and development (de Lucas, Daviere et al. 2008; Feng, Martinez et al. 2008). This research showed that DELLAs inhibit transcriptional activity of the PIF3 and PIF4 factors, two bHLH proteins reported to play a role in PHYB signalling, by binding the DNA recognition domain of these factors to form an inactive complex unable to bind to DNA. A similar inhibitory mechanism, mediated via interference of protein-protein or DNA binding ability of the interacting partners, was subsequently reported for other interacting proteins such as the JAZs (Hou, Lee et al. 2010). In this work, we set to analyze the functional implications of an additional interaction identified in the yeast two-hybrid screen, that of DELLAs with the ELF3 protein. This nuclear protein had been reported to function as a growth repressor and to modulate entrainment of the clock in response to various environmental inputs like light and temperature, in addition to play a role in gating light-regulated responses mediated by the clock (Covington, Panda et al. 2001; Liu, Covington et al. 2001; Carre 2002; Thines and Harmon 2010). The endogenous clock is tightly connected to the regulatory networks controlling cell elongation, what determines a rhythmic pattern of growth in young seedlings. This rhythmicity responds to internal cues, but is also regulated by external cues such as the length of the photoperiod, or the light quality/ intensity (Nozue, Covington et al. 2007; Niwa, Yamashino et al. 2009). The PIF factors are now accepted to be one of the main regulators of hypocotyl growth and to function as direct integrators of environmental light signals. The molecular mechanisms underlying rhythmic growth regulation by these factors, however, were totally unknown by the time this work was started (Nozue, Covington et al. 2007; Niwa, Yamashino et al. 2009; Leivar and Quail 2011). Noteworthy, *elf3* loss-of-function mutants had been described to display arrhythmic growth (Nozue, Covington et al. 2007), hence

highlighting ELF3-DELLA interaction as a possible mechanism for rhythmic growth regulation, by connecting the endogenous oscillator with growth. The elongated phenotypes of *elf3* and DELLA *global* mutants, however, were indicative of a negative regulatory function of these two proteins towards the PIF factors, hence pointing to a more complex model of regulation for these genes. As described below, we observed that over-expression of the ELF3 protein in the *PIF4*ox background, suppresses the elongated phenotype of these plants, hence pointing to a direct genetic interaction of these two genes. Therefore, in a first set of studies, we focused to investigate the regulatory effects of the ELF3 protein on PIF4 activity and thus in growth.

PIF4 and *PIF5* are functionally related and act downstream of *ELF3.*

The PIF4 factor was isolated in a mutagenesis screen for seedlings with an altered response to red light. Loss-of-function mutations in this gene lead to a short phenotype in light, and this phenotype is enhanced by subsequent mutation of the *PIF5* homologue, in *pif4pif5* seedlings (Lorrain, Allen et al. 2008; Niwa, Yamashino et al. 2009). Functional studies of these bHLH factors revealed that they act as positive regulators of hypocotyl growth in darkness, by playing a redundant function in seedling etiolation (Huq and Quail 2002; Khanna, Huq et al. 2004; Leivar, Monte et al. 2008; Lorrain, Trevisan et al. 2009; Leivar and Quail 2011). Lines over-expressing the *PIF4* gene were shown to have an elongated phenotype in continuous red light (cR), and to display arrhythmic growth under photoperiodic conditions (Huq and Quail 2002; Nozue, Covington et al. 2007), with multiple lines of evidence confirming that these factors play a pivotal role in hypocotyl growth (Leivar and Quail 2011). Loss-of-function mutations in the *ELF3* gene as in the *elf3-8* mutant, by contrary, lead to increased hypocotyl elongation and arrhythmic growth, in opposite to lines over-expressing this gene, which are shorter than the wild-type. Thus, these phenotypes identified the ELF3 protein as a growth repressor (Hicks, Albertson et al. 2001; Liu, Covington et al. 2001; Nozue, Covington et al. 2007).

To test a possible functional relationship between these proteins, that suggest a function of these genes in the same regulatory pathway, we crossed the *pif4pif5* mutant with plants carrying the loss-of-function *elf3-8* mutation, and *PIF4*ox plants (Khanna, Huq et al. 2004; Nozue, Covington et al. 2007) with *elf3-8* mutant and *35S::ELF3* transgenic lines (*ELF3ox*). Homozygous lines were selected in the F3 progeny from these crosses and hypocotyl lengths were measured from day 2 to day

5th post-germination, by growing these seedlings under photoperiodic (12L/12D) conditions (Figure 8a, b). Hypocotyls of these different genotypes were considered to be different when the means of the measured values were statistically significant using a t-student test. As seen in Figure 8b, hypocotyls lengths were already different by day 3 after germination and differences were even larger by day 5, in which hypocotyl elongation is considered to stop. As described, *elf3-8* seedlings showed in these studies an elongated phenotype, whereas *pif4pif5* plants were shorter than the *Col-0* wild type (Figure 8c). Interestingly, the triple *elf3-8pif4pif5* mutant displayed identical short hypocotyls as the *pif4pif5* mutant (Figure 8c), indicating an epistatic effect of the *pif4pif5* mutation over *elf3-8*.

Noteworthy, this phenotype suggests a role of the PIF4 and PIF5 factors downstream of ELF3, with transcriptional activity of these factors required for enhanced growth of *elf3-8* seedlings. *PIF4*ox lines, on the other hand, were taller than *elf3-8* mutants, and this mutation had a synergistic effect on PIF4 over-expression, as evidenced by the much taller phenotype of *elf3-8PIF4*ox seedlings compared to *PIF4*ox parental lines (Figure 8c). This effect suggests an interaction of these proteins, confirmed also by the intermediate hypocotyl lengths of *ELF3oxPIF4ox* lines compared to the *PIF4*ox and *ELF3*ox parental lines (Figure 8c).

Although these results would point to a role of ELF3 in modulating PIF4 stability, we could not exclude an additional regulatory effect of this protein at the transcriptional level, since *PIF4ox* lines express the *PIF4* gene under control of its own promoter. Levels of the *PIF4* transcript were actually reported to be increased by about 25-fold in *PIF4*ox lines, due to insertion of this extra gene copy close to an enhancer element or to the promoter region of an additional gene (Khanna, Huq et al. 2004; Nozue, Covington et al. 2007). Further analyses to test whether this transgene still responds to the environmental cues driving *PIF4* regulated expression, however, were not conducted. Therefore, we designed two sets of studies to establish if the observed hypocotyl growth effects of *ELF3*ox and the *elf3-8* mutation were actually mediated by transcriptional regulation of this transgene, or reflect a function of the ELF3 protein in modulating PIF4 protein activity.

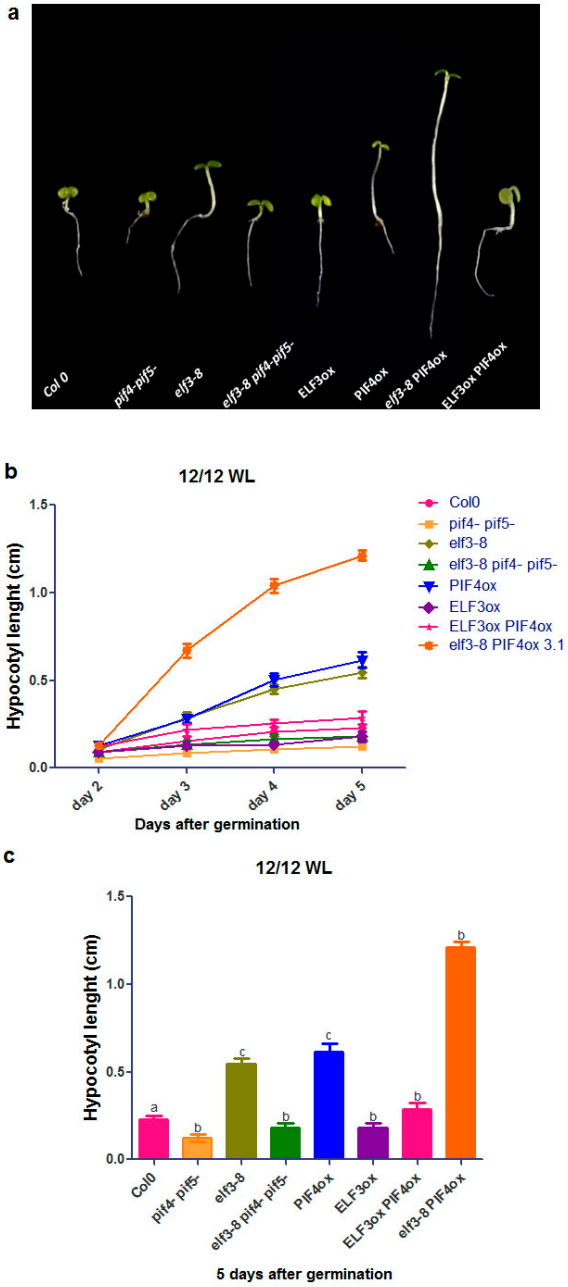

Figure 8. PIF4 and ELF3 have antagonistic roles on hypocotyl elongation. The *pif4pif5* mutation is epistatic to *elf3-8*, hence pointing to a role of these genes in the same signaling pathway.
a) Representative plants showing the hypocotyl lengths of these genotypes after 5 days of germination. b) Measures of the hypocotyl lengths of these plants, at different days after germination. c) Average hypocotyl lengths by 5 days of germination. Plants were grown in vertical plates on MS medium, under 12L/12D photoperiodic conditions. Represented are the means and SD of the measured lengths. Different letters denote statistical significant differences (*P*val<0.05) between genotypes, after applying the t-student test. The experiment was repeated three times with n=15 plants for each genotype, with identical results.

To assess a possible role of ELF3 in *PIF4* transcriptional regulation, *PIF4* expression profiles were analyzed in *elf3-8* and *ELF3ox* plants and levels of this transcript compared to those of *PIF4*ox plants, as described in the next section. A role of ELF3 in modulating *PIF4* transcriptional activity was also analyzed by investigating interaction of these proteins in yeast cells.

ELF3 regulates *PIF4* expression.

PIF4 plays an important role in diurnal regulation of hypocotyl growth under photoperiodic conditions, especially in short days (SD, 8h light, 16 dark) (Niwa, Yamashino et al. 2009). The elongated hypocotyl of *elf3-8* loss-of-function mutations, on the other hand, is associated to an arrhythmic pattern of growth of these seedlings that were shown to also elongate during early night, a phase of the day at which *PIF4* is usually repressed (Nozue, Covington et al. 2007). *elf3-8* mutants are actually impaired in diurnal expression of several clock and flowering time genes (Kim, Hicks et al. 2005; Yu, Rubio et al. 2008; Thines and Harmon 2010), the finding that mutations in the *PIF4* and *PIF5* genes are epistatic to this mutation (Figure 8) thus pointing to a role of ELF3 in diurnal expression of these factors. *PIF4* transcripts were shown to accumulate during late night, to peak at day time and be down-regulated at dusk (Nozue, Covington et al. 2007). Interestingly, the diurnal pattern of oscillation of this gene is anti-phasic with that of *ELF3* (Mockler, Michael et al. 2007) (http://diurnal.mocklerlab.org), which is suggestive of an antagonistic function of these proteins (Figure 9a). PIF4 activity, on the other hand, seems to be restricted to the end of the night phase, as in the light, this factor is rapidly destabilized by phytochromes, which promote phosphorylation and subsequent proteasomal degradation of the protein (Al-Sady, Ni et al. 2006; Castillon, Shen et al. 2007). Hence, rhythmic hypocotyl growth appears to be defined by the phase of accumulation of this factor (Nozue, Covington et al. 2007; Niwa, Yamashino et al. 2009). The ELF3 protein, in turn, has been reported to follow a similar pattern of accumulation as its transcript, with a peak of expression around ZT12, independent of photoperiodic conditions (Yu, Rubio et al. 2008).

To verify if ELF3 plays a role in transcriptional regulation of the *PIF4* gene, *PIF4* transcript levels were analyzed by qPCR in 4 day old *Col-0*, *elf3-8* and *ELF3ox* plants, grown in short days (8L/16D). Samples were collected every 4 hours for a total period of 24 hours, with the first sample collected when lights were turned off. ZT0 was defined as the time of the day at which lights are on. As show in Figure 9b, the *PIF4* transcript followed a different diurnal pattern of accumulation in *elf3-8* seedlings compared to *Col-0* and *ELF3*ox plants. In the *Col-0* and *ELF3*ox backgrounds expression of this transcript is down-regulated to basal levels during the night, to start accumulating by the end of the night phase and reach maximal expression levels during the light phase (ZT24 to 32 in Figure 9b). In *elf3-8* seedlings, by contrary, *PIF4*

did not show a rhythmic pattern of expression, with elevated levels of this transcript detected during the whole night phase (ZT8 to 20 in Figure 9b). This finding would suggest that ELF3 represses *PIF4* transcription early in the night, although this protein in not required for activation of this gene, since similar levels of transcript are detected in all three genetic backgrounds during day time. Interestingly, *PIF4* repression was also found to be stronger in *ELF3*ox lines, during the first half of the night, consistent with a role of the ELF3 protein in *PIF4* repression at this specific phase of the day (ZT8 to 16).

The *PIF4* factor has been reported to activate gene expression by binding conserved G-box and E-box (CATGTG) elements in the promoters of its target genes. *PIL1* and *XTR7* are direct targets this factor, as the promoters of these two genes were recovered in PIF4 chromatin immunoprecipitation (ChIP) assays (de Lucas, Daviere et al. 2008; Lorrain, Allen et al. 2008). Therefore, we decided to analyze if diurnal expression of these genes is as well altered in *ELF3* lines, in a correlated manner with misregulated expression of the PIF4 factor. As shown in Figures 9c and d, *PIL1* and *XTR7* transcript were found to accumulate to much higher levels in *elf3-8* seedlings, with differences respect to WT or *ELF3*ox plants being particularly marked during early night (ZT12 to 24). Interestingly, these transcripts are also higher during the light phase, although during this interval of the day *PIF4* transcript levels were similar in all three genotypes. The peak of *PIL1* and *XTR7* transcripts, at the end of the night, was also slightly reduced in *ELF3*ox seedlings which, with exception of this small change showed a similar pattern of accumulation of these transcripts as the wild-type. Together, these observations confirm a role of ELF3 in transcriptional repression of the *PIF4* gene during early night. However, the fact that over-expression of this protein does not lead to a strong repression of *PIF4* transcription suggest that the presence of other regulators, in addition to the ELF3, is required to repress expression of this gene. In agreement with this notion, ELF3 has been very recently shown to bind the ELF4 and LUX proteins to form the so called evening complex (EC), an inhibitory complex that represses *PIF4* expression and that of several other morning-phased genes, to function as one of the inhibitory loops of the clock (Nusinow, Helfer et al. 2011).

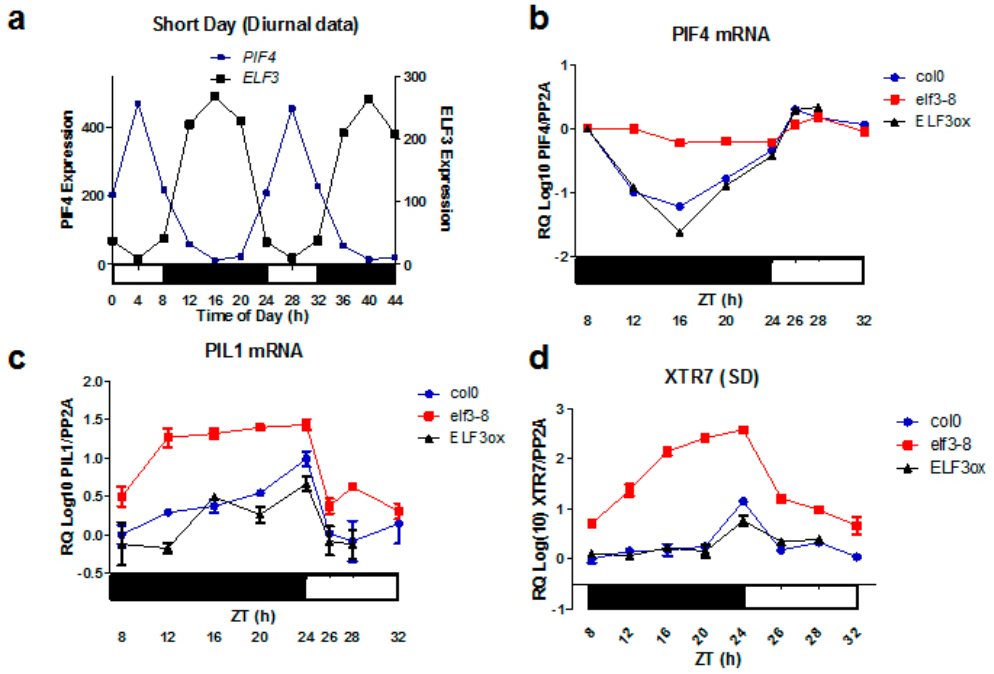

Figure 9. ELF3 modulates *PIF4* diurnal expression and affects expression of its downstream regulated targets. a) The expression profiles of the *ELF3* and PIF4 genes are anti-phasic in short day conditions, according to data retrieved from the DIURNAL database (diurnal.mocklerlab.org). b) The *PIF4* transcript is misregulated in *elf3-8* mutants. Impaired regulation of this gene leads to an altered pattern of expression of its direct gene targets, c) *PIL1* and d) *XTR7,* with these transcripts accumulating to higher levels. Data are the means and SD of three technical replicates. The experiment was repeated three times with similar results.

Loss-of-function mutation of the *ELF3* gene, in the *elf3-8* mutant, impairs function of this complex and de-represses *PIF4* expression during early night. This has a marked effect on expression of the *PIL1* and *XTR7* gene targets, as the PIF4 protein is stabilized at night. However, ELF3 regulatory effects seem not to be restricted to this phase of the day, since *elf3-8* lines show also elevated levels of the *PIL1* and *XTR7* transcripts during the light phase, even during this interval of the day *PIF4* transcript levels are not sensibly different from wild-type plants, (Figure 9b-d). Also, it is remarkable that *ELF3ox* lines show shorter hypocotyls than the *WT* (Figure 8c) not only when grown under photoperiodic conditions, but also under continuous monochromatic red light, where stability of the PIF4 protein is expected to be limiting (Liu, Covington et al. 2001; Kim, Hicks et al. 2005). This suggests that ELF3 may regulate PIF4 activity by additional mechanisms than just its function as a component of the evening complex (EC) repressing expression of this gene.

Hypocotyl elongation correlates with *PIF4* expression levels.

Under photoperiodic conditions, seedlings exhibit a rhythmic pattern of growth which, in short days, is characterized by low elongation rates during the whole day and early night, and by a rapid elongation growth by the end of the night (Nozue, Covington et al. 2007; Niwa, Yamashino et al. 2009). Therefore, we decided to analyze levels of accumulation of the *PIF4* transcript and its downstream target *PIL1,* in the ELF3/PIF4 lines we had generated. To that aim, 5 day old seedlings grown under short days were collected at ZT22 for qPCR analyses. At this point of the day, the rate of growth elongation is maximal (Nozue, Covington et al. 2007), and in *Col-0* the levels of *PIF4* and *PIL1* transcripts are elevated (Figure 9c, (Nozue, Covington et al. 2007; Lorrain, Allen et al. 2008)). As seen in Figure 10, *PIF4* expression was highest in both *PIF4*ox and *elf3-8 PIF4*ox lines, while *ELF3oxPIF4ox* showed lower levels of this transcript than *elf3-8* seedlings. *ELF3*ox plants showed reduced *PIF4* transcript levels, while intermediate levels of this transcript were detected in *ELF3oxPIF4ox* lines, likely due to EC repression of the native promoter driving expression of this transgene.

Regarding *PIL1* gene expression (Figure 10b), comparable levels of this transcript were detected in *elf3-8*, *PIF4*ox and *elf3-8PIF4*ox seedlings, suggesting that levels of the PIF4 factor might be saturating in these plants or that additional factors are also activating expression of this gene. PIFs for instance were shown to have highly redundant functions (Leivar, Monte et al. 2008), with PIF3 and PIF7 also shown to function at *PIL1* activation (Li, Ljung et al. 2012; Soy, Leivar et al. 2012).

Interestingly, these two PIF factors are atypical, since *PIF3* does not a diurnal regulation pattern and PIF7 is not destabilized by phytochromes (Li, Ljung et al. 2012; Soy, Leivar et al. 2012). Thus, ELF3 might play a more general role in regulating PIFs transcriptional activity than just at *PIF4* repression. *PIL1* transcript levels, on the other hand, were reduced to similar levels in *ELF3*ox and *ELF3oxPIF4ox* plants, although the later accumulated higher levels of the *PIF4* transcript than *ELF3*ox plants, thus pointing to a role of ELF3 in regulating PIF4 transcriptional activity at the protein level. *pif4pif5* and *elf3-8pif4pif5* seedlings, in addition, showed the lowest levels of *PIL1* gene expression, thus corroborating a major role of the PIF4 and PIF5 factors in direct transcriptional regulation of this gene. To gain further insights on the functional role of ELF3 in transcriptional regulation of the *PIF4* gene and the potential role of this protein in regulating PIF4 transcriptional activity, additional gene expression studies were also conducted using *pPIF4*::LUC and *pPIF4*::PIF4-LUC lines.

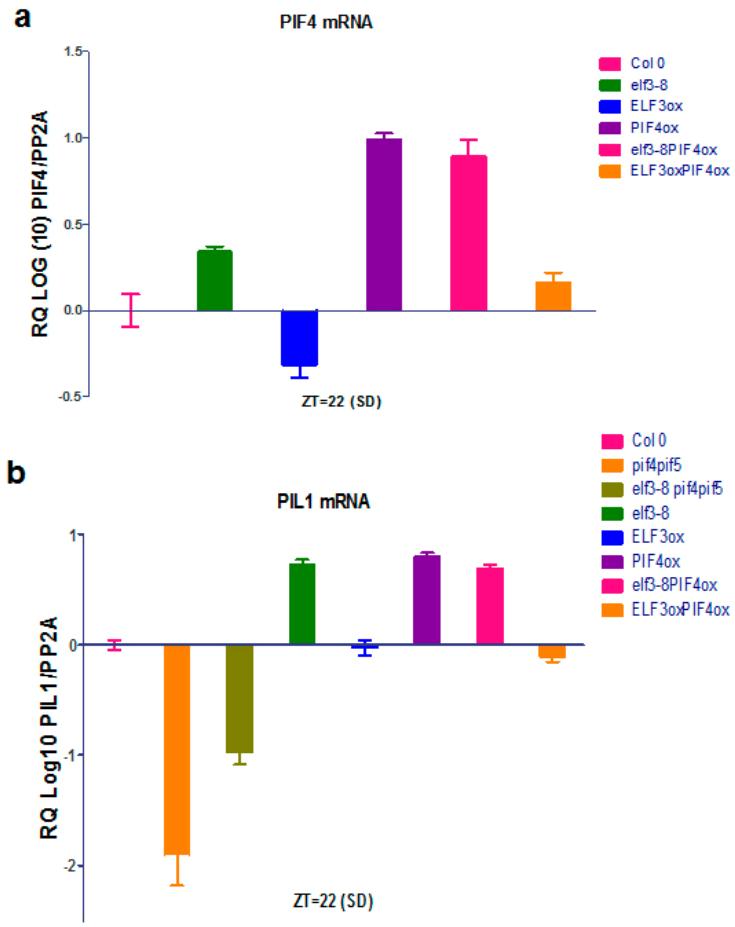

Figure 10. Correlation of *PIF4* expression levels and downstream activation of its direct *PIL1* gene target. Expression of these genes was analyzed at ZT22, when seedling elongation growth is maximal. a) *PIF4* expression levels correlate with the hypocotyl lengths of these seedlings, shown in Figure 8. b) *PIL1* transcript levels correlate with *PIF4* expression, although point to a role of ELF3 in regulation of PIF4 transcriptional activity at the protein level. Data are the means and SD of three technical replicates

PIF4 expression studies using luciferase reporter constructs.

In order to analyze *PIF4* diurnal expression, by means of less time consuming assays, transgenic lines bearing the *PIF4* promoter fused to the luciferase (*LUC*) reporter (*pPIF4*::LUC) were generated. To track levels of accumulation of the protein, an additional construct was also generated, expressing a LUC reporter fusion of this protein under control of its own promoter (*pPIF4*::PIF4-LUC). A fusion of the downstream target *PIL1* promoter to the LUC gene (*pPIL1*::LUC) was as well used to estimate transcriptional activity of the accumulating PIF4 protein. Transgenic seeds bearing this construct were kindly provided by Joanne Chory's lab, with these plants

being recently described in (Li, Ljung et al. 2012). With this method, named *LucTrap* system, diurnal profiles of endogenous clock and output genes have been recently analyzed (Calderon-Villalobos, Kuhnle et al. 2006), largely contributing to expand our understanding of the molecular mechanisms regulating the internal oscillator. By measuring luciferase activity of these different lines we were able to track *PIF4* promoter activation, PIF4 protein levels and PIF4 transcriptional activity. To this aim, single seedlings were transferred to 96 plate wells, the luciferin substrate was added, and LUC activity was measured in these plants during several days. At least 12 independent seedlings were used for each genotype or treatment, statistical analyses of the data thus providing a more robust estimation of the respective expression profiles. Plants were grown for 3 days under short day photoperiodic conditions before being transferred to the plates. They were allowed to acclimate for at least 12 hours, before luciferase activity was recorded during successive days.

As shown in Figure 11a, luciferase activity driven by the *pPIF4*::LUC construct showed a similar rhythmic pattern of expression as reported in the DIURNAL database or detected in our qPCR studies (Figure 9a,b). It is important to remark that the luciferase system involves an enzymatic reaction that provides a remarkable sensitivity to the method, less than the qPCR reaction but similar to that of a northern blot (Calderon-Villalobos, Kuhnle et al. 2006). Luciferase activity of *pPIF4*::LUC seedlings increased late in the night to peak at the end of the light phase and start decreasing to reach basal levels by the middle of the night (Figure 11a). Surprisingly, luciferase activity driven by the *pPIF4*::PIF4-LUC construct followed a similar diurnal pattern as the observed for *pPIF4*::LUC seedlings, although a more prominent peak of LUC activity was observed by the end of the night, hence reflecting an increased stability of the protein at this phase of the day (Figure 11a). Activity was drastically reduced after dark to light transition, to be subsequently restored during the rest of the day/early night, to lead to a similar profile as seen for the promoter fusion. This pattern of expression indicates that whereas the protein is stable in the dark, it is rapidly destabilized in the light, in particular during the first hours after dark to light transition. Subsequently, the protein follows a similar pattern of accumulation as the own transcript, hence denoting a transient accumulation of the newly synthesized protein, before being destabilized in the light.

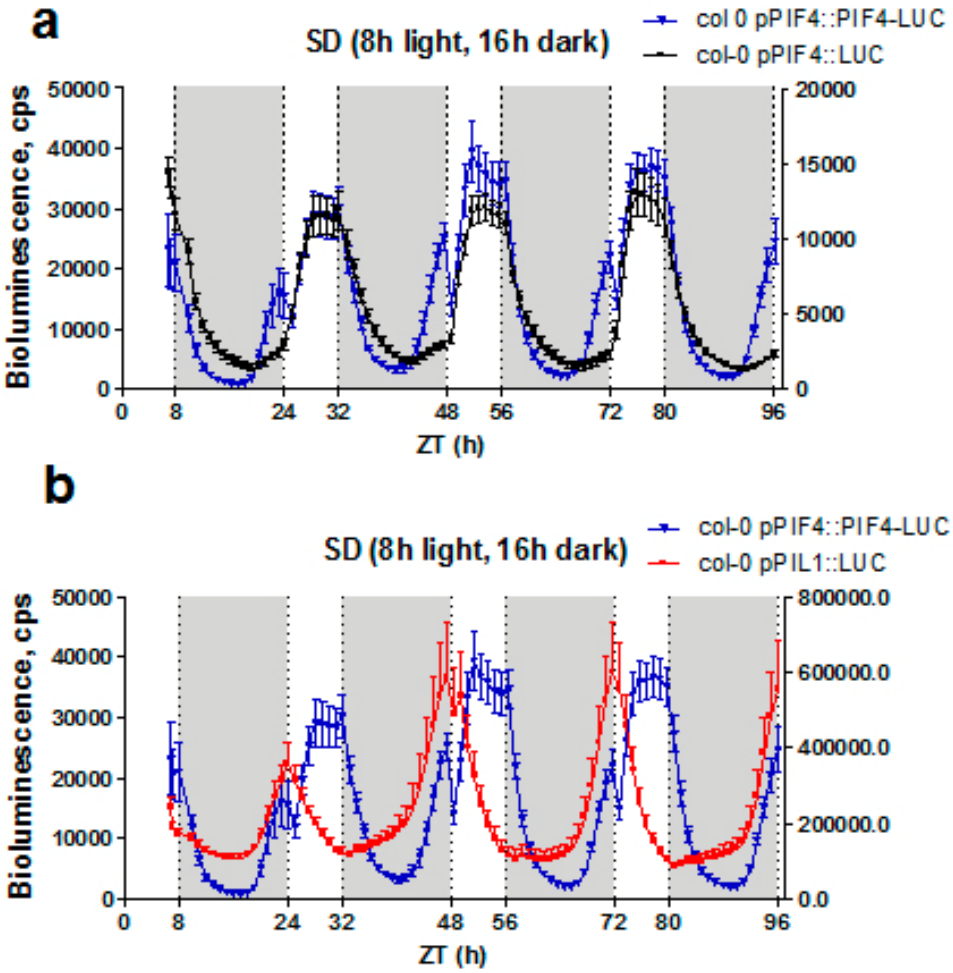

Figure 11. Diurnal levels of luciferase activity in seedlings expressing the *pPIF4*::LUC, *pPIF4*::PIF4-LUC and *pPIL1*::LUC constructs. a) The *PIF4* promoter shows in short days a rhythmic pattern of expression, as reported. The PIF4 protein is stabilized at the end of the night. b) PIF4 stabilization coincides with the peak of expression of the *pPIL1*::LUC construct. Data are the means and SEM of at least 15 plants. The experiment was repeated three times with identical results.

Such a transient accumulation is in apparent contradiction with several reports pointing to a rapid destabilization of the PIF4 protein in the light (Castillon, Shen et al. 2007; Nozue, Covington et al. 2007). At present, we cannot to rule out that fusion to the 61 kD LUC protein, interferes with normal light degradation of this protein, by impairing interaction with the proteolytic machinery or with additional factors triggering PIF4 degradation in the light. However, rapid degradation of this fusion protein was still observed shortly after transition to light. In addition, these seedlings did not show any elongated phenotype that could suggest an abnormal accumulation of the PIF4-LUC

fusion protein. Hitherto, all PIF4 protein stability studies have been conducted using constitutive promoter fusions of this protein (Nozue, Covington et al. 2007; de Lucas, Daviere et al. 2008; Lorrain, Allen et al. 2008) and therefore, may not fully reproduce stability of this factor. Noteworthy, the end of the night peak of accumulation of the PIF4 protein, coincides with the peak of activity of the *pPIL1*::LUC construct (Figure 11b), and with the period where the PIF4 factor has been reported to be transcriptionally active. This indicates that with this system we can actually follow diurnal expression of these genes and have a good estimation of the protein accumulation levels and PIF4 transcriptional activity. Therefore, these lines were crossed to *elf3-8* and *ELF3ox* plants to assess *PIF4* transcription and protein levels in these genetic backgrounds. Homozygous lines were selected from these crosses, and luciferase activity was measured as before (Figure 12).

As seen in Figure 12a, *pPIF4*::LUC luciferase activity was higher in *elf3-8* compared to the *ELF3*ox and *Col-0* backgrounds. Interestingly, in *elf3-8* plants luciferase activity remained high during early night, as seen in Figure 9b for the *PIF4* transcript, hence evidencing a misregulated expression of the *PIF4* gene in darkness. In *ELF*3ox plants, in opposite, a more abrupt decrease in luciferase activity was observed during early night, indicative of a stronger repression of this gene in the dark. Higher levels of luciferase activity were also observed during the day in *ELF3*ox lines compared to the *Col-0* wild-type, hence leading to higher amplitude of oscillation in these plants (Figure 12a). However, as *ELF3*ox has larger cotyledons (Figure 40), we cannot exclude that this effect is caused by biased detection of LUC activity in these organs.

Interestingly, levels of luciferase activity driven by the *pPIF4*::PIF4-LUC construct were significantly higher in *elf3-8* seedlings compared to *Col-0* and *ELF3ox* plants (Figure 12b). LUC activity after dark transition was also increased in these plants compared to *elf3-8 pPIF4*::LUC seedlings, hence suggesting a lower rate of degradation of the protein during this phase of the day. Nonetheless, further studies will need to be conducted to verify that this construct faithfully reproduces PIF4 protein levels, before any conclusion is derived. Even so, luciferase activity driven by the *pPIL1*:LUC construct was also much higher in *elf3-8* seedlings than in *ELF3*ox and *Col-0* plants (Figure 12c), hence evidencing that the *elf3-8* mutation strongly promotes PIF4 transcriptional activation of this gene.

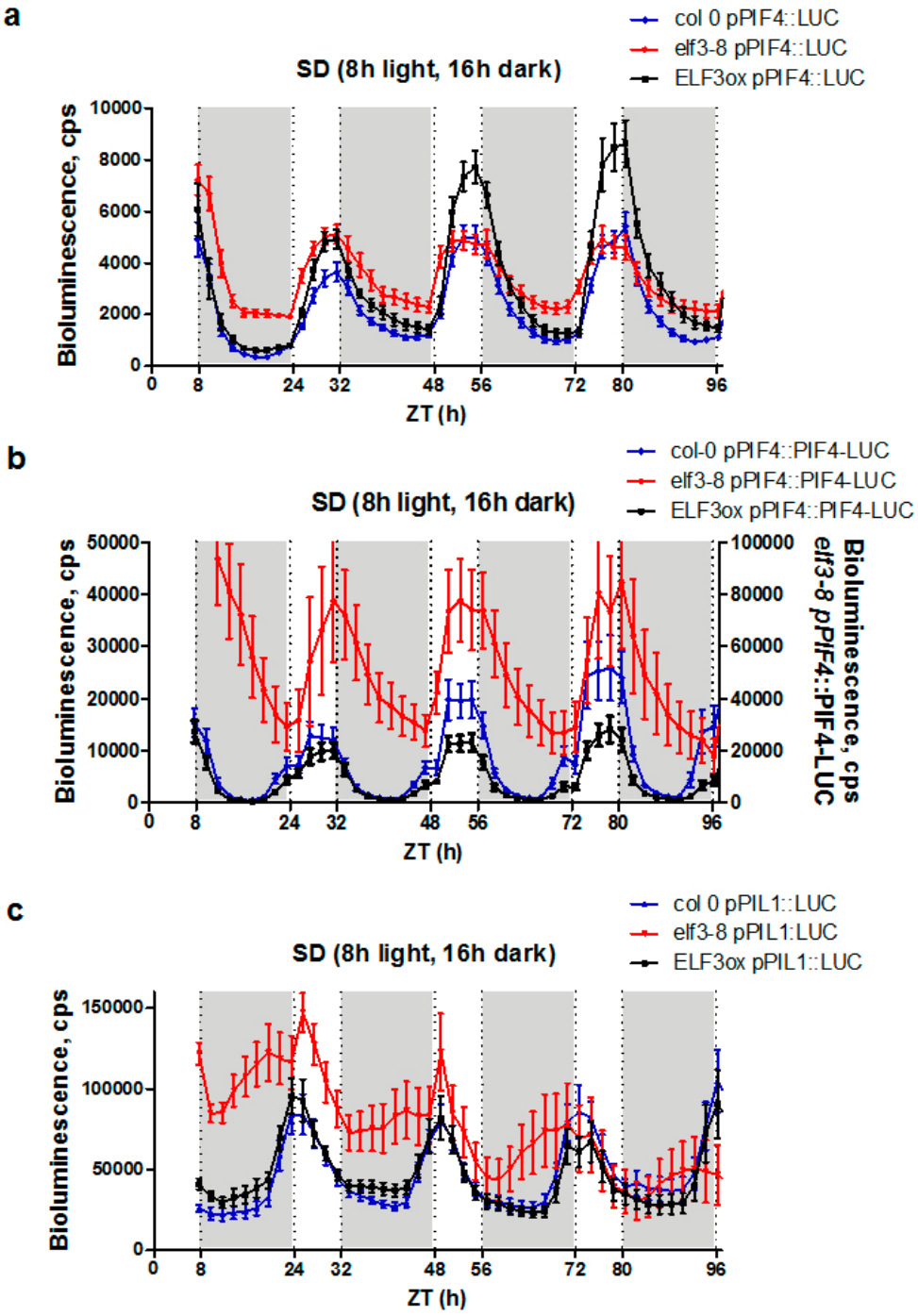

Figure 12. LUC activity driven by the *pPIF4*::LUC, *pPIF4*::PIF4-LUC and *pPIL1*::LUC constructs in the ELF3 lines. a) *PIF4* is misregulated in *elf3-8* mutant plants. b) The PIF4 protein appears to be strongly stabilized in *elf3-8* mutant seedlings. c) PIF4 stabilization correlates with a strong activation of the *pPIL1*::LUC construct in the *elf3-8* mutant. Data are the means and SEM of at least 15 plants.

Together, these results validate the *pPIF4*::LUC and *pPIL1*::LUC constructs as useful tools to analyze *PIF4* gene expression and transcriptional activity of this factor, although suitability of the *pPIF4*::PIF4-LUC construct needs to be further assessed. Therefore, these lines should enable testing interaction of the ELF3 and PIF4 proteins under a broader range of conditions, to provide a better understanding of the molecular mechanisms implicated in ELF3 regulation of PIF4 transcriptional activity.

At this point of the work, results reported by the group of Steve Kay in the 21st International Conference of Arabidopsis Research (Steve A. KAY 2010) and later published in (Nusinow, Helfer et al. 2011), established that ELF3 represses *PIF4* gene expression at the transcriptional level, hence in part confirming our results. By screening a transcription factor library for circadian clock regulators, in combination with tandem mass spectrometry identification of the proteins co-purified with the ELF3 tagged protein, these authors showed that ELF3 binds the ELF4 and LUX proteins to form a ternary complex, named the evening complex (EC), that represses expression of morning-phased genes during early night (Steve A. KAY 2011). Expression of these three proteins coincides at dusk, with ELF3 binding both ELF4 and LUX proteins to recruit them into this negative regulatory complex. Binding of this complex to the promoters of the morning-phased genes is mediated by the LUX transcription factor, with several LUX binding sites being found to be present in the promoters of the *PIF4* and *PIF5* genes. LUX was actually found to mediate EC complex interaction with the *PIF4* and *PIF5* promoters, hence repressing expression of these factors during early night, to gate the hypocotyl growth response during this phase of the day (Nusinow, Helfer et al. 2011). In agreement with this model, the *PIF4* and *PIF5* genes are misregulated in the *elf3-1*, *elf4-3* and *lux-4* mutants that display elongated phenotypes. The *pif4pif5* mutation, on the other hand, is epistatic to the loss-of-function mutations in any of these genes, hence confirming a function of the PIF4 and PIF5 factors downstream of the EC repressive complex (Nusinow, Helfer et al. 2011).

This model accounts for several of our previous results, but still does not provide a good explanation to the much higher levels of LUC activity observed in *elf3-8 pPIF4*::PIF4-LUC lines compared to *elf3-8 pPIF4*::LUC plants. Enhanced LUC activity of the *pPIF4*::PIF4-LUC and p*PIL1*:LUC constructs in the *elf3-8* mutant background actually suggests an additional function of the ELF3 protein in modulating PIF4 stability/transcriptional activity and in consequence, we set to assay if these proteins are able to interact in yeast two hybrid cells as well as in the plant.

ELF3 interacts with the PIF4 factor in yeast cells.

To test interaction of these proteins, the ELF3 coding region was amplified by PCR out of *Col-0* plants and cloned into the pGBKT7 vector, to be used as bait. An already available PIF4 construct in the pGADT7 vector (de Lucas, Daviere et al. 2008) was used as prey in these assays. As shown in Figure 13b, these proteins were found to interact in yeast cells, since cells co-transformed with these two constructs grow in selective media, while the respective controls transformed with the empty vectors not (Figure 13b).

Amino acid sequence comparison of members of the PIF family identified two main conserved domains in the PIF4 protein, the APB domain that is involved in PHYB interaction, and the bHLH domain that mediates protein dimerization and binding of this factor to the promoters of its target genes (Huq and Quail 2002; Khanna, Huq et al. 2004). Results by our group, also identified the bHLH domain as the domain that mediates interaction with the DELLAs, showing that complex formation with these repressors blocks this factor DNA binding ability (de Lucas, Daviere et al. 2008). Deletions generated in that work (Figure 13a), were used for interaction studies with the ELF3 bait construct, to define which region of the PIF4 protein binds ELF3. As seen in Figure 13c, the N-terminal region containing the APB domain (del1) did not lead any viable cell in the selection media and thus is unable to interact with ELF3. Similar results were also obtained for the C-terminal half of the protein (del4), which covers the region just after the bHLH domain to the C-terminal end. However, a positive interaction with the ELF3 bait construct was observed for the other two deletions, from the start codon to the bHLH domain (del2) or from the bHLH domain to the C-end of the protein (del3), indicating that the bHLH domain mediates ELF3 interaction. As discussed above, this domain was found to mediate interaction with the DELLAs (Castillon, Shen et al. 2007; de Lucas, Daviere et al. 2008; Hornitschek, Lorrain et al. 2009), and is required for dimerization and interaction with other atypical HLH factors (Huq and Quail 2002; Castillon, Shen et al. 2007) that, like the DELLAs, inhibit DNA recognition and thus transcriptional activity of the PIF4 protein (de Lucas, Daviere et al. 2008; Hornitschek, Lorrain et al. 2009; Hao, Oh et al.). Interaction with this domain hence suggests a similar ELF3 regulatory mechanism as seen for the DELLAs, with interaction with this protein being likely to block PIF4 DNA binding ability.

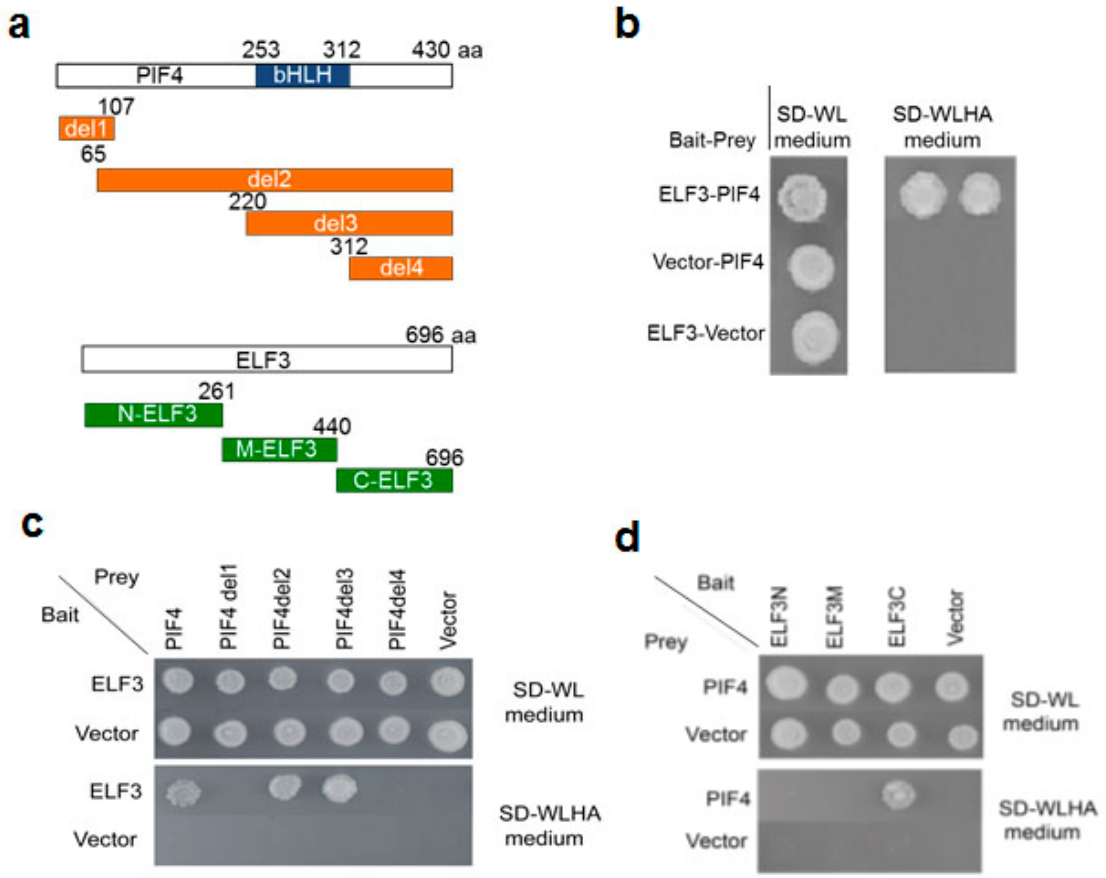

Figure 13. Yeast two-hybrid assays for interaction of the ELF3 and PIF4 proteins. a) Protein deletions used to map the interaction domains. b) ELF3 interacts with the PIF4 factor in yeast cells. c) Deletions of the PIF4 protein that contain the bHLH domain were able to bind the ELF3 bait construct. d) The ELF3 C-terminal domain mediates interaction with the PIF4 protein. AH109 yeast cells co-transformed with the bait and prey constructs, or with the corresponding empty vector, used as negative control, were grown on SD-WL media as a control for co-transformation and on SD-WLHA selection media, to assay for protein interaction.

Alignment of the ELF3 protein homologues from different plant species, on the other hand, identified three highly conserved domains in these proteins (Figure 13a) (Liu, Covington et al. 2001; Herrero, Kolmos et al. 2012). Up to date, a function has been associated to only two of these domains, the N-ELF3 domain involved in the interaction with PHYB and the COP1 E3 ubiquitin ligase, and the M-ELF3 domain which has recently been reported to bind the EC ELF4 protein (Liu, Covington et al. 2001; Yu, Rubio et al. 2008; Nusinow, Helfer et al. 2011; Herrero, Kolmos et al. 2012). The C-terminal end of the protein (C-ELF3) includes a putative NLS and dimerization domain (Liu, Covington et al. 2001), and was shown to be needed for ELF3 localization in

nuclear speckles. However, no interaction partners were identified that associate to this region (Herrero, Kolmos et al. 2012). To test if any of these domains is required for interaction with PIF4, deletions of the ELF3 protein encompassing these domains were generated (Figure 13a) and cloned with the Gateway system into the PGBKT7 vector, for transformation with the PIF4-pGADT7 prey construct. As shown in Figure 13d, neither the N-ELF3 nor the M-ELF3 constructs led to viable cells in the selection media and therefore fail to interact with PIF4. Instead, a positive interaction was observed for the C-ELF3 construct, hence pointing to a role of the ELF3 C-terminal end in PIF4 interaction. Interestingly, this factor is the first interaction partner described to bind this region.

ELF3 interacts with other members of the bHLH PIF family.

The DNA recognition bHLH region is highly conserved among all members of the PIF family as well as other bHLH factors (Toledo-Ortiz, Huq et al. 2003). Therefore, if ELF3 binds to this region possibly interacts with other members of the PIF family. From previous published work (Gallego-Bartolome, Minguet et al. 2010), several pGADT7 constructs for different bHLHs were available in the lab. From these, we selected PHYTOCHROME INTERACTING FACTOR 3-like 5 (*PIF1/PIL5*) a close relative of PIF4 that plays an important role as a negative regulator of seed germination and chlorophyll biosynthesis (Oh, Kim et al. 2004), LONG HYPOCOTYL IN FAR-RED (*HFR1*), an atypical non DNA binding HLH that lacks the conserved basic domain (Fairchild, Schumaker et al. 2000) and functions as a negative modulator of PIFs by forming non DNA-binding heterodimers with a role in far-red and blue signaling (Fairchild, Schumaker et al. 2000; Duek and Fankhauser 2003; Hornitschek, Lorrain et al. 2009) and SPATULA (*SPT*), a light-stable repressor of seed germination, that mediates germination in response to cold temperatures, and also represses vegetative growth at low temperatures (Penfield, Josse et al. 2005; Sidaway-Lee, Josse et al. 2010).

As seen in Figure 14, all these bHLHs were shown to bind ELF3 in yeast cells, although this interaction needs to be confirmed *in vivo*. Interestingly, the HFR1 factor, that lacks a conserved basic region, shows a stronger binding affinity, hence indicating that ELF3 binds the HLH domain in these proteins. This finding raises the question of whether ELF3 is implicated in the regulation of these factors. To the date, function of these regulators has not been associated to that of ELF3, neither ELF3 was reported to

play a role in seed germination (Oh, Kim et al. 2004). Interestingly, HFR1 is induced by shade and far-red light and ELF3 has been identified as a QTL for shade-avoidance (Jimenez-Gomez, Wallace et al. 2010). SPT, on the other hand, shows a circadian expression pattern but stability of this factor is not regulated by phytochromes (Josse, Gan et al. 2011). This factor has been reported to be co-regulated with FHY3, FAR1, CCA1, LHY and HY5 (Li, Siddiqui et al. 2011) and thus it is possible that it modulates entrainment of the clock in response to cold temperatures, with ELF3 playing a role in gating this response.

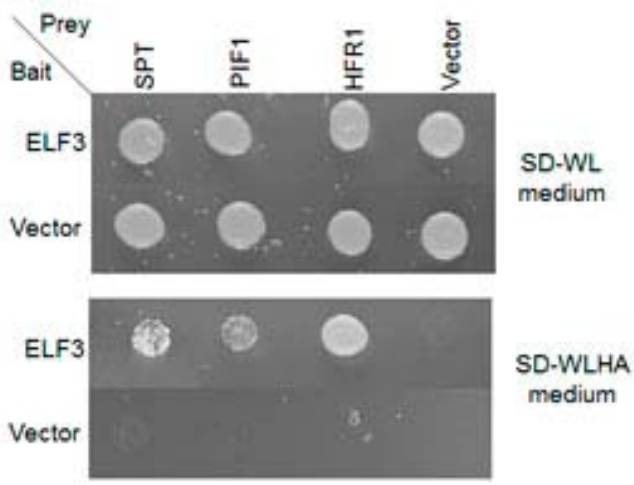

Figure 14. Yeast two-hybrid interaction of ELF3 and members of the PIF family of bHLHs. ELF3 binds the bHLH domain that is highly conserved in all members of this gene family. An positive interaction is observed for these three PIF proteins, including the HFR1 factor that lacks a conserved basic region. This indicates that ELF3 interaction is mediated by the HLH domain.

In-vivo interaction of the ELF3 and PIF4 proteins

The finding that ELF3 binds the HLH domain of the PIF4 factor points to a role of this protein in repressing PIF4 transcriptional activity, by competing this factor for binding to the promoters of its regulated genes. A similar mechanism of regulation has actually been reported for the DELLAs as well as for other atypical bHLHs, or PHYB (de Lucas, Daviere et al. 2008; Hornitschek, Lorrain et al. 2009; Galstyan, Cifuentes-Esquivel et al. 2011; Park, Park et al. 2012). Before verifying this negative regulatory model, interaction of these proteins was confirmed *in planta*. For this, tagged versions of these proteins were expressed in *N. benthamiana* leaves and used in co-immunoprecipitation assays (Liu, Zhang et al. 2010) . This system overtakes the limitations of *in-vitro* systems, such as the lack of required protein modifications, is much faster than generating stably transformed plants, and still closely resembles physiological conditions, since additional regulatory partners should be present in the tobacco cells.

To this aim, epitope tagged versions of these proteins, under control of the constitutive

35S promoter, were generated. *ELF3* was cloned into the pYL-CFP vector (gift from Vicente Rubio) to fuse the CFP epitope tag to the C-terminal end of the protein, while *PIF4* was cloned into the pGWB14 vector to generate a C-terminal fusion of this protein to the HA epitope. These constructs were transformed into *Agrobacterium* and co-infiltrated in *N. benthamiana* leaves, to test for interaction. After two days of infiltration, protein extracts were obtained from the leaves, immunoprecipitated with GFP conjugated agarose beads, and detected by western blot with the GFP and HA antibodies, to test if the PIF4-HA protein was present in the immunoprecipitated fraction. As shown in Figure 15a, a band corresponding to the PIF4-HA protein was detected after ELF3-CFP in GFP agarose immunoprecipitation, hence confirming interaction of these proteins in plant cells. This band was not observed in a negative control using extracts expressing only the PIF4-HA protein and equally immunoprecipitated with GFP agarose beads, thus demonstrating that this immunoreactive band is not due to unspecific binding to the beads (Figure 15a).

Identical results were obtained in a reverse assay where extracts were immunoprecipitated with anti-HA conjugated beads and the pulled-down ELF3-CFP protein analyzed by western blot detection with an anti-GFP antibody (Figure 15b). As before, no immunoreactive band was detected after immunoprecipitation of extracts of plants expressing only the ELF3-CFP protein, used as negative controls.

Interaction of these proteins was also confirmed by BiFC (Bi-molecular complementation assay) studies in which fusions of these proteins to the nYFC and cYFC fragments of the fluorescent YFP protein were co-expressed in *N. benthamiana* leaves. A nuclear fluorescence signal was detected in leaves co-expressing both protein fusions but not in the negative controls expressing one of these fusions and the corresponding empty vector (Figure 15c). Thus, we can conclude that the ELF3 and PIF4 proteins interact *in planta.*

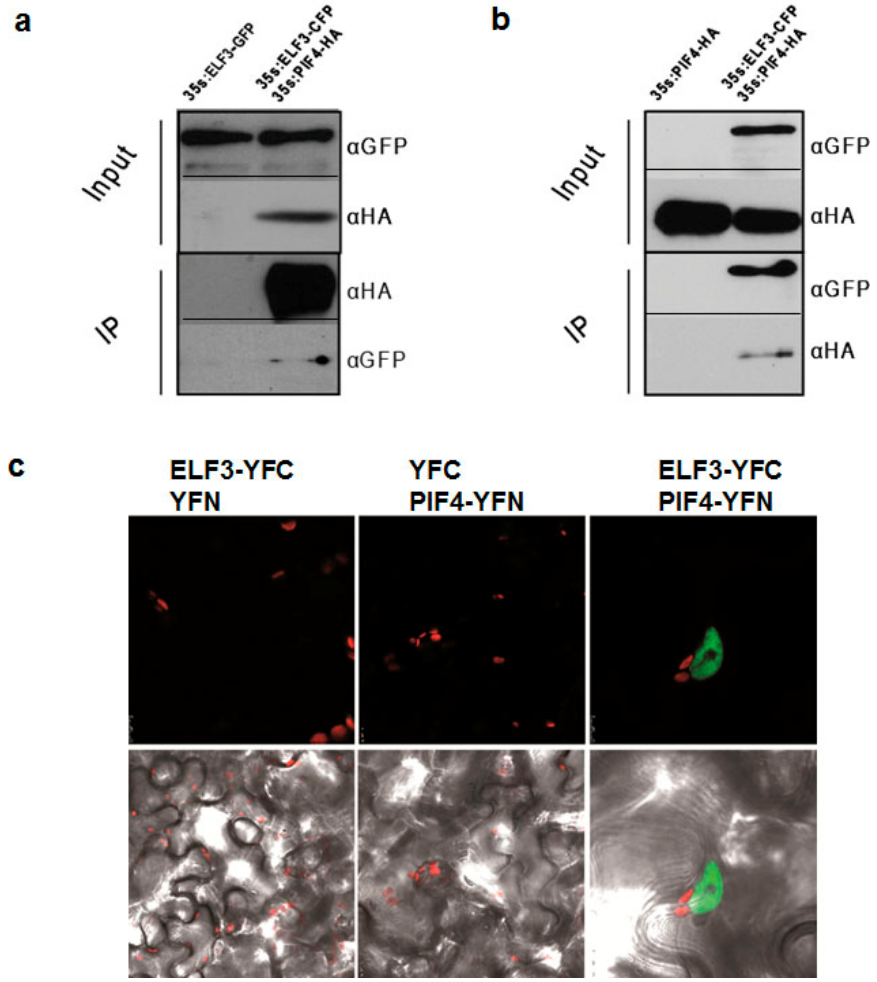

Figure 15. Co-immunoprecipitation and BiFC analyses of PIF4/ELF3 interaction in *N. benthamiana* leaves a) The PIF4-HA protein is pulled-down by immunoprecipitation of the ELF3-CFP protein out of extracts of leaves infiltrated with both protein constructs. A band corresponding to this protein is not detected when extracts of leaves infiltrated only with the PIF4-HA construct are incubated with the anti-GFP beads. b) Identical results are obtained when these assays are carried out in a reverse orientation. The ELF3-CFP protein is pulled-down by immunoprecipitation of the PIF4-HA protein with anti-HA beads, but this band is not detected in when ELF3-CFP infiltrated leaves are used as negative control. c) Nuclear YFP fluorescence detected in leaves infiltrated with the nYFC-PIF4 and cYFC-ELF3 constructs. YFP fluorescence was not detected in leaves infiltrated with these constructs and the corresponding empty vectors.

ELF3 inhibits PIF4 transcriptional activity in transient assays.

The PIF4 factor activates expression by binding to G-box elements in the promoters of its gene targets (de Lucas, Daviere et al. 2008; Lorrain, Allen et al. 2008; Kunihiro, Yamashino et al. 2011; Hornitschek, Kohnen et al. 2012). The *PIL1* gene is known to be a direct target of this factor, as a region of this promoter including two G-box elements is highly enriched in chromatin immunoprecipitation studies with the PIF4 protein (Hornitschek, Lorrain et al. 2009). Therefore, this promoter was cloned in the LucTrap3 vector (Calderon-Villalobos, Kuhnle et al. 2006) to generate the *pPIL1*::LUC reporter construct used in transactivation assays. The *35S*::PIF4-HA and *35S*::ELF3-HA fusions were used as effector constructs (Figure 16). For transactivation studies, *N. benthamiana* leaves were infiltrated with *Agrobacterium* cultures for these different constructs. An *Agrobacterium* strain bearing the pBINAR empty vector was used to normalize bacterial cell concentration, when only the reporter or just one of the effector constructs was infiltrated. Co-expression of the *35S*::ELF4-HA construct was also used as negative control, to assess that expression of multiple constructs does not have an interfering effect on activation. As shown in Figure 16a, expression of the *35S*::PIF4-HA effector construct resulted in strong activation of the *pPIL1*::LUC reporter, compared to the leaves infiltrated with the reporter construct alone. Expression of the *35S*::ELF3-HA construct, by contrary, failed to activate *pPIL1*::LUC expression, indicating that ELF3 is unable to activate this gene. Levels of expression of this reporter, in addition, were reduced when both *35S*::ELF3-HA and *35S*::PIF4-HA effectors were co-expressed, indicating that ELF3 has an inhibitory effect on PIF4 transcriptional activity (Figure 16a). Such an inhibitory effect was not observed when this factor was co-expressed with the control *35S*::ELF4-HA effector (Figure 16c), hence demonstrating that ELF3 repression is not caused by competition or silencing of the different *35S* effector constructs. Western blot studies (Figure 16c) actually showed that levels of expression of the PIF4 and ELF3 proteins are very similar in leaves infiltrated with either one or with both effector constructs, hence excluding silencing effects.

From these results we can conclude that ELF3 inhibits transcriptional activity of the PIF4 factor, by binding the HLH domain and hindering interaction with the promoter region of the *PIL1* gene. This same mechanism was reported for the DELLAs (de Lucas, Daviere et al. 2008), with co-expression of these repressors also leading to inhibition of PIF4 transcriptional activity in transient activation assays (Figure 16d).

Hence, our results agree with those of Nusinow *et al.* (Nusinow, Helfer et al. 2011) pointing to a role of ELF3 in transcriptional repression of the *PIF4* gene, but add an additional layer of regulation, by showing that these proteins directly interact and that interaction of these proteins inhibits PIF4 transcriptional activity. Further evidence in this direction is also shown in the next section.

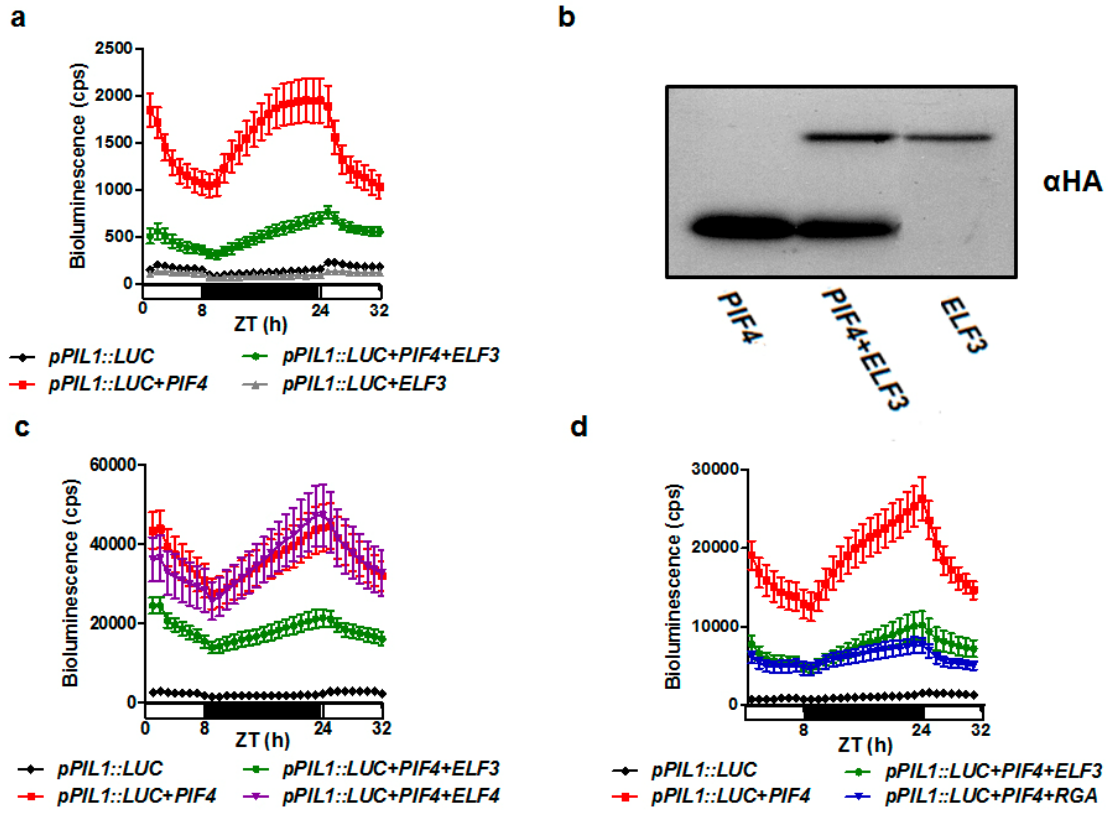

Figure 16. Transactivation assays in *N. benthamiana* leaves demonstrate a repressive function of ELF3 on PIF4 transcriptional activity. a) Co-expression of ELF3 inhibits *pPIL1*::LUC activation by the *35S*::PIF4-HA effector construct. b) Similar levels of these proteins are observed in leaves expressing either the *35S*::PIF4-HA and *35S*::ELF3-HA effector constructs or co-infiltrated. c) Co-expression of the 35S::ELF4-HA as an effector does not lead to reduced levels of expression of the *pPIL1*::LUC reporter, excluding competition or silencing effects between both *35S* promoters. c) RGA expression leads to a similar inhibition of LUC activity as seen for the ELF3 protein, these two proteins sharing a similar mechanism of repression. Data are the means and SEM of at least 16 leaf discs. These experiments were repeated three times with similar results.

Repression of PIF4 transcriptional activity is independent of transcriptional regulation of this gene.

A principal feature of the clock is its ability to buffer responses to external inputs, gating acute response to these signals during certain intervals of the day. ELF3 is a component of one of the clock loops and as such plays an important role in this buffering capacity, which relies on a complex interaction of both input and output clock responses (McWatters, Bastow et al. 2000; Covington, Panda et al. 2001; Carre 2002; Thines and Harmon 2010; Dixon, Knox et al. 2011; Kolmos, Herrero et al. 2011). For several of these responses, complex feed-back mechanisms have been uncovered, which implicate transcriptional regulatory loops and protein-protein interaction mechanisms found to modulate key clock components activity. CCA1, for instance, has been shown to bind the *ELF3* promoter to repress transcription of this gene (Lu, Webb et al. 2012), with these two proteins being also shown to interact in yeast cells, although biological relevance of this interaction is still unknown (Yoshida, Fekih et al. 2009). GA regulation of PIFs activity is also mediated through transcriptional regulatory and protein-protein interaction events. PIF1, for example, binds the *RGA* and *GAI* promoters to repress seed germination (Oh, Kim et al. 2004), at the same time that these repressors block PIFs transcriptional activity to restrain growth (de Lucas, Daviere et al. 2008). A further example of complex regulation is that reported for the control of PIFs stability by phytochrome, i.e. PHYB-mediated degradation of these factors triggers the shift from skotomorphogenesis to photomorphogenesis, but PIF4, at the same time, has been reported to facilitate PHYB degradation by the E3 ligase COP1 (Jang, Henriques et al. 2010). PHYB, in addition, was recently reported to inhibit PIF4 DNA interaction and thus transcriptional activity of this factor (Park, Park et al. 2012). Thus, the finding that ELF3 regulates PIF4 activity at both the transcriptional and protein levels is not totally odd, although it will be important to assess during which interval of the day this protein-protein control becomes relevant.

This is not trivial, since both levels of regulation are expected to co-exist. However, under free running conditions, components of the EC complex are expected to no longer oscillate, enabling to test functional relevance of this protein-protein control. Thus, additional studies were conducted by growing seedlings under continuous red light (cR) and analyzing p*PIL1*::LUC activity after transfer to FR light conditions, known to stabilize the PIF4 protein (Castillon, Shen et al. 2007). Studies using simulate shade conditions (WL+FR) actually showed that activation of the *ATHB2*, *ATHB4*, *PAR1*, and

PIL1 genes does not require *de-novo* protein synthesis (Lorrain, Trevisan et al. 2009), indicating that stabilization of the PIF4 factor is sufficient to mediate activation of these genes (Lorrain, Trevisan et al. 2009; Kunihiro, Yamashino et al. 2011). With respect to the ELF3 protein, there are no reports concerning expression of this gene or stability of the protein under FR light. Allelic variants of this gene were shown to be responsible of the QTL associated to the differential response to shade of the *Bay* and *Sha Arabidopsis* ecotypes (Jimenez-Gomez, Wallace et al. 2010; Coluccio, Sanchez et al. 2011), indicating that the protein is still functional in FR-enriched light.

Hence, homozygous p*PIL1*::LUC lines in the *Col-0*, *elf3-8, and ELF3*ox backgrounds were grown in cR light, to follow activation of this gene upon a shift to FR light. LUC activity was measured in a luminometer as before, and recorded with intervals of 1 hour, for the 4 hours preceding transfer to FR light, and 12 additional hours after the FR light switch. LUC levels of p*PIF4*::LUC lines in these genetic backgrounds were also measured, to verify that the switch to FR light does not lead to transcriptional activation of the *PIF4* gene. As seen in Figure 17a, luciferase activity of p*PIF4*::LUC lines was not sensibly modified (a small repression is observed) in response to FR treatment, evidencing that the switch to FR does not induce *PIF4* gene expression. In opposite, a notable induction of the p*PIL1* promoter was observed in response to this treatment, with a much stronger peak of activation seen in the *elf3-8* mutant compared to the other backgrounds (Figure 17b). Yet, since basal levels of expression of the p*PIF4*::LUC construct are also higher in this mutant, we cannot exclude that this up-regulated response is not due to higher levels of accumulation of the PIF4 factor in these seedlings. However, activation of the p*PIL1*::LUC gene is clearly reduced in *ELF*3ox plants, even though p*PIF4*::LUC expression is basically identical or, if any, slightly increased in these plants with respect to the wild-type (Figure 17a).

By qPCR studies, we also verified that expression of the *ELF3* gene is not sensibly modified in response to FR light (Figure 17c), hence demonstrating that the ELF3 protein represses *PIL1* activation in FR, independently of *PIF4* transcription, with direct interaction of the ELF3 and PIF4 proteins thus playing a role in gating acute response to FR light.

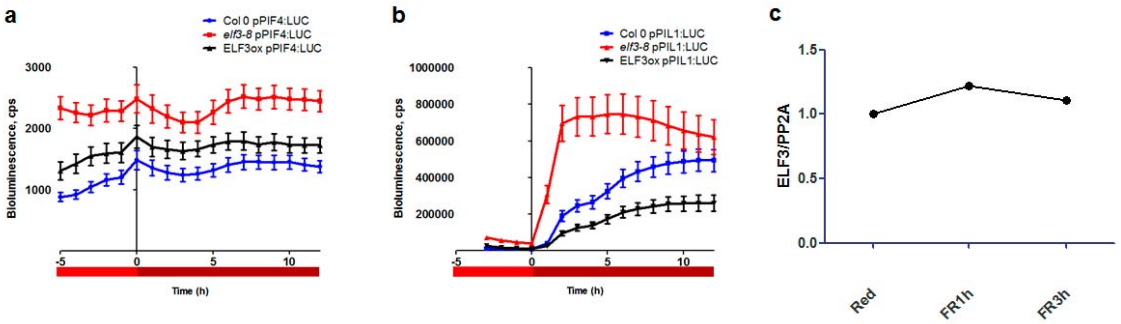

Figure 17. ELF3 inhibits PIF4 transcriptional activity in red to far-red treatments. a) *PIF4* transcription is not modified by the switch to FR. b) FR up-regulation of p*PIL1*::LUC transgene is reduced in *ELF3*ox plants, while *elf3-8* mutants show much stronger up-regulated levels of expression of this gene. c) Activation of the *PIL1* gene is not associated to transcriptional repression of the *ELF3* gene. p*PIL1*::LUC and p*PIF4*::LUC activities are the means and SEM of at least 24 seedlings. *ELF3* expression was analyzed by qPCR. Data are means and SD of three technical replicates.

Since LUC assays were found to be less sensitive than qPCR studies, we also verified these results by qPCR. Seedlings were collected just before the shift from cR to FR light, and after 1 hour and 3 hours of far-red treatment for RNA extraction and qPCR. As seen in Figure 18, qPCR data for *PIF4* and *PIL1* expression were consistent with those of the LUC assays (Figure 18) with *PIF4* transcript levels showing only marginal changes in response to the FR light treatment, although transcript levels are higher in *elf3-8* seedlings compared to the other backgrounds (Figure 18a). The *PIL1* transcript is also up-regulated to much higher levels in the *elf3-8* mutant than in wild-type, while *ELF3*ox plants show reduced levels of activation of this gene (Figure 18b). Basically, similar results were obtained for other PIF4 regulated genes, although for these genes profiles in the *elf3-8* and *ELF3*ox backgrounds were not as different as seen for the *PIL1* gene. For *XTR7* (Figure 18c), gene activation is delayed compared to that of *PIL1*, but up-regulation is still stronger in *elf3-8* mutants and reduced in *ELF3*ox plants. Activation of the *IAA19* and *ATHB2* genes was found to be transitory, with lower levels of these transcript observed in samples collected after 3 hours of FR light treatment compared to those of 1 hour of FR light (Figure 18d,e). Expression profiles of these genes were also different in the sense that feed-back regulation of the *IAA19* transcript was less prominent in *elf3-8* mutants that in the wild-type and *ELF3*ox backgrounds, hence implicating ELF3 function in feed-back regulation of this gene. The *ATHB2* (Kunihiro, Yamashino et al. 2011) transcript, by contrary, showed increased levels of

expression in the *elf3-8* mutant, by 1h and 3 hours of FR light treatment, and also reduced levels of expression in *ELF3*ox plants at both time points, hence suggesting that feed-back regulation of this gene does not require ELF3 function (Figure 18d). Together, these results confirm a role of ELF3 in gating FR-activated responses mediated by stabilization of the PIF4 factor, although the different profiles of these gene targets suggest a more complex regulation of the *IAA19* and *ATBH2* genes.

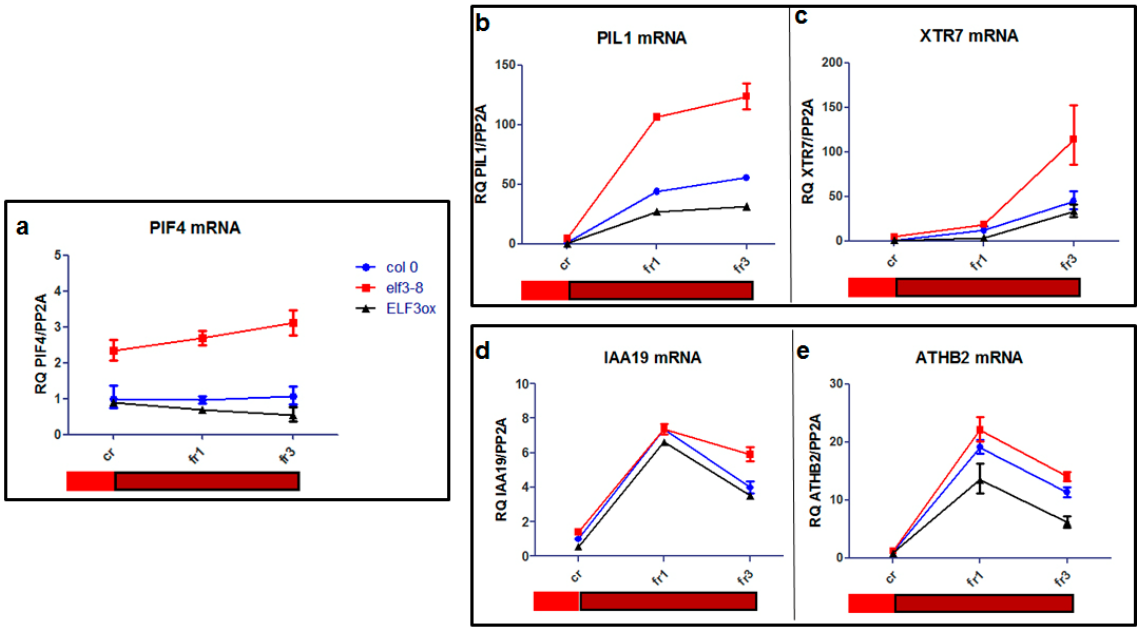

Figure 18. ELF3 modulates PIF4 transcriptional activity upon transfer from cR to FR light. In continuous light, expression of the evening complex (EC) components does not oscillate and diurnal expression of the *PIF4* gene is lost. Transfer to FR light leads to PHYB inactivation and PIF4 protein stabilization a) *PIF4* rate of expression is not sensibly changed by the shift from cR to FR light, changes in downstream gene expression reflecting changes in PIF4 transcriptional activity. b-e) Expression profiles of the *PIL1*, *XTR7*, *IAA19* and *ATBH2* genes. Levels of these transcripts are increased in *elf3-8* mutants and reduced in *ELF3*ox plants, hence evidencing a role of the ELF3 protein in direct regulation of PIF4 transcriptional activity. The IAA19 and ATBH2 transcripts are only transiently induced in response to FR light, denoting a more complex regulation of these genes. Data are the

PIF4 is transcriptionally active in cR light.

Data shown in Figures 18b-e were normalized to T0 expression levels, before the switch to FR light. However, levels of expression of these transcripts in cR were different in the *elf3-8* and *ELF3*ox backgrounds (Figure 19). These differences are likely to be associated to the higher *PIF4* transcription rates in the *elf3-8* genotype, although increased PIF4 transcriptional activity may also contribute to such different

basal expression. PIF4 protein is thought to be destabilized in cR, still differences are observed in the downstream *PIL1* target which to some extent correlate with PIF4 expression, indicating that PIF4 actively transcribes this gene also in cR light (Figures 19a, b).

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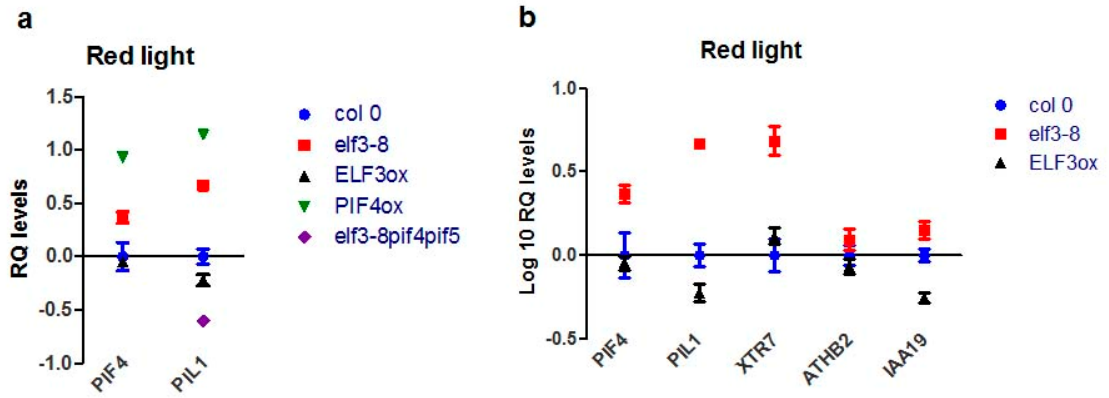

Figure 19. PIF4 still regulates gene expression in cR light. Although it is widely accepted that the PIF4 factor is destabilized in the light, enhanced transcription of this gene in the *elf3-8* mutant, leads to higher levels of expression of its gene targets. a) Levels of expression of the *PIL1* gene are elevated in cR in the *elf3-8* mutant and *PIF4*ox plants, activated expression of this gene hence mirroring *PIF4* transcript levels b) Basal gene activation is not identical for all *PIF4* target genes, with *elf3-8* seedlings showing increased levels of the *PIL1* and *XTR7* transcripts but not of that of the *ATHB2* and *IAA19*, which are only transiently activated in response to FR light. Data are the means and SD of three technical replicates.

Interestingly, such increase in basal expression levels is not observed for all PIF4 gene targets, with similar levels of expression of the *ATHB2* and *IAA19* genes observed in *elf3-8* seedlings as in wild-type plants (Figure 19b). These transcripts were found to be transiently induced in response to FR light, and thus they are not only regulated by PIF4, but also by other regulators. These genes are actually involved in auxin and brassinosteroid signaling (Kolmos, Herrero et al. 2011; Zhou, Song et al. 2012) and they may be subjected to negative feed-back regulation by these pathways.

From these results we can conclude that PIF4 transcriptional activity is not totally repressed in continuous red light and this factor is likely to play a role in gene regulation in light grown seedlings. Additionally, the profiles of activation of the different target genes are not identical, indicating a complex regulation depending on light conditions, intrinsic each gene.

The shift to FR light stabilizes PIF4 and decreases ELF3 protein levels.

In the red to far red studies, the *elf3-8* mutants showed clearly enhanced levels of activation of the PIF4 gene targets, but repression of these genes in *ELF3*ox lines was observed to be small compared to the wild-type (Figures 18, 19). By qPCR studies we verified that *ELF3* expression is not down-regulated in response to the FR light treatment (Figure 17c), the mild phenotype of *ELF3*ox lines hence suggesting a low stability of the ELF3 protein in FR light. Hence, we decided to analyze levels of accumulation of this protein, by using an anti-ELF3 specific antibody generated against the C-terminal region of the protein (Nusinow, Helfer et al. 2011). Transgenic p*PIF4*::PIF4-HA lines were also used to correlate ELF3 with PIF4 protein levels, since an antibody to the PIF4 protein is not available. Remarkably, these lines display a wild-type phenotype and thus accumulate normal levels of this protein.

pPIF4::PIF4-HA, *elf3-8* and *ELF3*ox seedlings were grown as before in cR light and samples were collected just before the switch, and 1 h and 3 hours after transferring them to FR light conditions, for western blot studies. As reported (Lorrain, Trevisan et al. 2009), FR light stabilized the PIF4 protein, with an accumulation of the PIF4-HA protein observed as early as 1 hour of FR light treatment and further increased by 3 hours of FR treatment. Noteworthy, although this factor has been reported to be phosphorylated in a PHYB-dependent manner and destabilized in the light (Khanna, Huq et al. 2004; Al-Sady, Ni et al. 2006; Castillon, Shen et al. 2007), we still detected a weak band of the PIF4-HA protein in cR light, indicating that a residual pool accumulates in continuous red light (Figure 20a). Interestingly, stabilization of the PIF4 protein in FR light correlates with ELF3 destabilization, with much lower levels of this protein being observed after 1 hour of FR light, to be no longer detected by 3 hours of FR treatment. This band is not observed in *elf3-8* extracts, confirming that it corresponds to the ELF3 protein. Also, intensity of this bans is much stronger in ELF3ox extracts, and likewise destabilized in FR light, although to a lower rate than in wild-type plants. Abundance of this protein actually increased by 1 hour of FR light treatment, to be destabilized by 3 hours of transfer to FR light (Figure 20b). We can conclude from these results that while the PIF4 protein accumulates in far-red light, ELF3 is stable in red light but rapidly destabilized under far-red conditions. Thus, degradation of this protein in FR light explains the relatively mild changes in gene expression seen in *ELF3*ox plants (Figure 18).

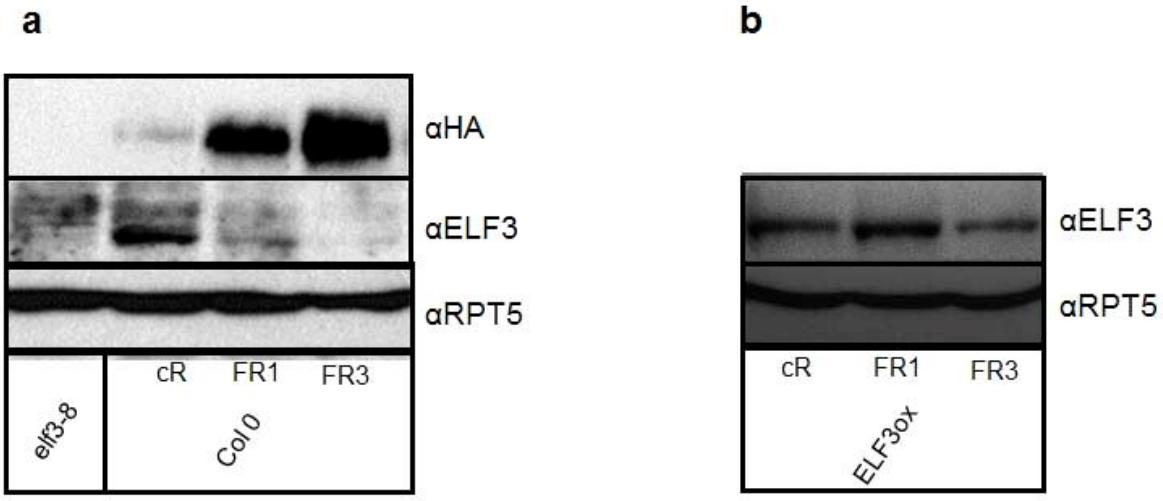

Figure 20. The PIF4 and ELF3 proteins show opposite patterns of accumulation in cR and FR lights. a) Levels of expression of the PIF4-HA fusion in transgenic p*PIF4*::PIF4-HA lines. The protein is stabilized by the shift to FR light. ELF3, by contrary, is stable in cR, but rapidly destabilized in FR light. The band for this protein is not detected in *elf3-8* extracts, thus confirming identity of this band as ELF3. b) Levels of accumulation of the ELF3 protein in transgenic *ELF3*ox plants. In these plants, rate of destabilization of the protein is slower than in the wild-type, with relatively high levels of the protein detected after 3 hours of shifting the plants to FR light. The western blots were incubated with an anti-HA antibody and with a specific antibody raised against the C-terminal region of the ELF3 protein. Incubation with the anti-RPT5 protein antibody was used as a loading control for these extracts.

PIF4 modulates ELF3 levels in red light.

The observation that the PIF4 and ELF3 proteins display an inverse pattern of accumulation suggests a role of these proteins in modulating each other stability. To confirm such negative correlation, we analyzed levels of the ELF3 protein in *pif4pif5* and *PIF4*ox seedlings grown in cR light. As seen in Figure 21a, higher ELF3 levels are actually detected in *pif4pif5* plants compared to the wild-type, while levels of this protein are reduced in *PIF4*ox lines. These differences are not associated with changes in *ELF3* gene expression (Figure 21b), hence demonstrating a role of the PIF4 factor in modulating ELF3 stability. Noteworthy, although *pif4pif5* mutant accumulate the ELF3 protein to higher levels in cR light, transfer of these seedlings to FR light still leads to ELF3 destabilization, indicating that other members of the PIF family may also play a role in modulating ELF3 stability, or that ELF3 destabilization in FR light is mediated by a different regulatory mechanism.

These findings point to a role of PIFs in controlling ELF3 abundance, at least in cR light, and consistent with this role, ELF3 protein levels were found to be further

increased in the different *pif* triple mutants and the quadruple *pifQ* mutant, especially for mutant combinations including the *pif3* allele (Figure 21c).

Noteworthy, further analyses of PHYB levels, using an anti-PHYB antibody, showed that this photoreceptor displays in cR light a similar pattern of accumulation as the ELF3 protein, with reduced PHYB levels in *PIF4*ox lines and higher levels of this photoreceptor in the *pif4pif5* mutant (Figure 21a).

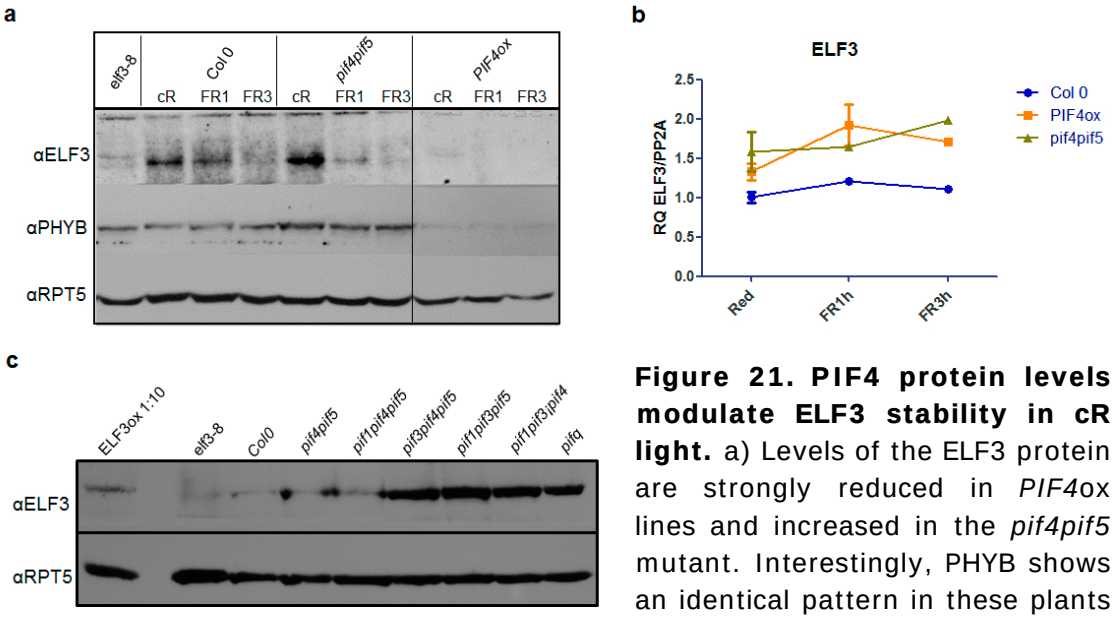

Figure 21. PIF4 protein levels modulate ELF3 stability in cR light. a) Levels of the ELF3 protein are strongly reduced in *PIF4*ox lines and increased in the *pif4pif5* mutant. Interestingly, PHYB shows an identical pattern in these plants as the ELF3 protein, suggesting that in cR light PIF4 modulation of ELF3 stability involves function of this photoreceptor mutant, suggesting that PIF4 modulation of ELF3 stability in cR light may also involve these photoreceptors function b) Expression of the *ELF3* gene is not affected in these genetic backgrounds. c) ELF3 protein levels in triple and quadruple PIF mutants suggesting a modulation of ELF3 stability by the PIF family, especially PIF3, in continuous red light. RPT5 was used as a loading control. qPCR data are means and SD of two biological replicates. Immunodetection with an anti-RPT5 antibody was used as a loading control.

Actually, ELF3 has been shown to bind PHYB and the E3 ligase COP1 through the same N-ELF3 domain (Liu, Covington et al. 2001; Yu, Rubio et al. 2008), with COP1 interaction recently reported to mediate proteasomal degradation of the ELF3 protein (Yu, Rubio et al. 2008). Thus, PIF4 might regulate ELF3 stability by modulating PHYB levels in cR light. In agreement with this hypothesis, PIF4, together with the PIF3 and PIF7 factors, were shown to regulate responses to prolonged red light by reducing PHYB levels, via a process thought to involve the proteasomal pathway (Leivar et al.,

2008). Therefore, we further analyzed abundance of the ELF3 protein in *PHYB*ox and *phyB* mutant lines. As shown in Figure 22, high levels of accumulation of this protein were observed in *PHYB*ox lines, whereas in *phyB* seedlings this protein is reduced to below detection levels. Hence, these results demonstrate a role of PHYB in ELF3 stabilization, suggesting that strong destabilization of this protein in *PIF4*ox lines may be mediated by increased turnover of the PHYB photoreceptor.

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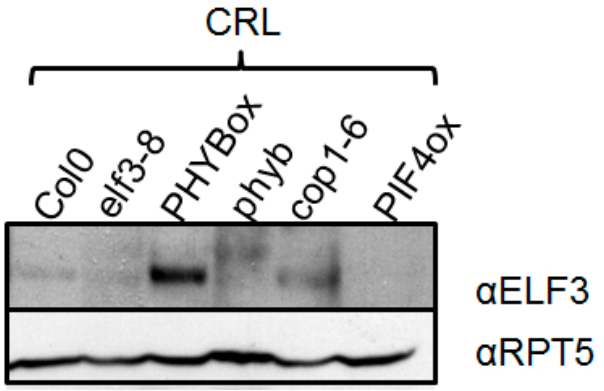

Figure 22. PHYB-dependent stabilization of ELF3.
*PHYB*ox lines accumulate increased levels of the ELF3 protein in cR light, while a immunoreactive band for this protein cannot be detected in *phyB* and *PIF4*ox lines. Incubation with the anti-RPT5 protein antibody was used to verify equal loading of these protein extracts.

To gain further evidence for an inverse correlation of these proteins, we analyzed PIF4 and ELF3 levels in plants grown under photoperiodic conditions, in a time window in which expression of these genes overlap. In short day conditions, the ELF3 protein was shown to peak around ZT12 (Nusinow, Helfer et al. 2011), whereas in *cop1-4* mutant seedlings this peak is shifted to the end of the day (ZT8), indicating that the COP1 E3 ligase plays an active role in destabilizing the ELF3 protein at dusk (Yu, Rubio et al. 2008). At ZT8, *PIF4* is expressed to high levels (Nozue, Covington et al. 2007; Nusinow, Helfer et al. 2011) (Figure 8) and although this protein is considered to be destabilized by light, low levels of the protein are still observed (Nozue, Covington et al. 2007). Therefore, we chose this time window to analyze ELF3 protein levels in *35S*::PIF4-HA transgenic lines. These lines were shown to segregate into tall and small plants, due to silencing events (Lorrain, Allen et al. 2008). Therefore, they provide an excellent background where to test an inverse correlation between ELF3 and PIF4 protein levels. Seedlings were cultivated under short day conditions and at ZT8, we collected those seedlings showing the characteristic tall phenotype of *PIF4*ox plants (named *Tall*) from those showing a shorter phenotype due to the silencing of the transgene (*Small*, Figure 23a). As shown in Figure 23b, we were unable to detect the PIF4-HA protein in extracts of *small* plants, hence demonstrating that the PIF4

transgene is silenced in these seedlings. Strikingly, levels of the ELF3 protein were found to be much higher in *small* plants (Figure 23b) than in *tall* seedlings accumulating high levels of the PIF4-HA protein (Figure 23b).

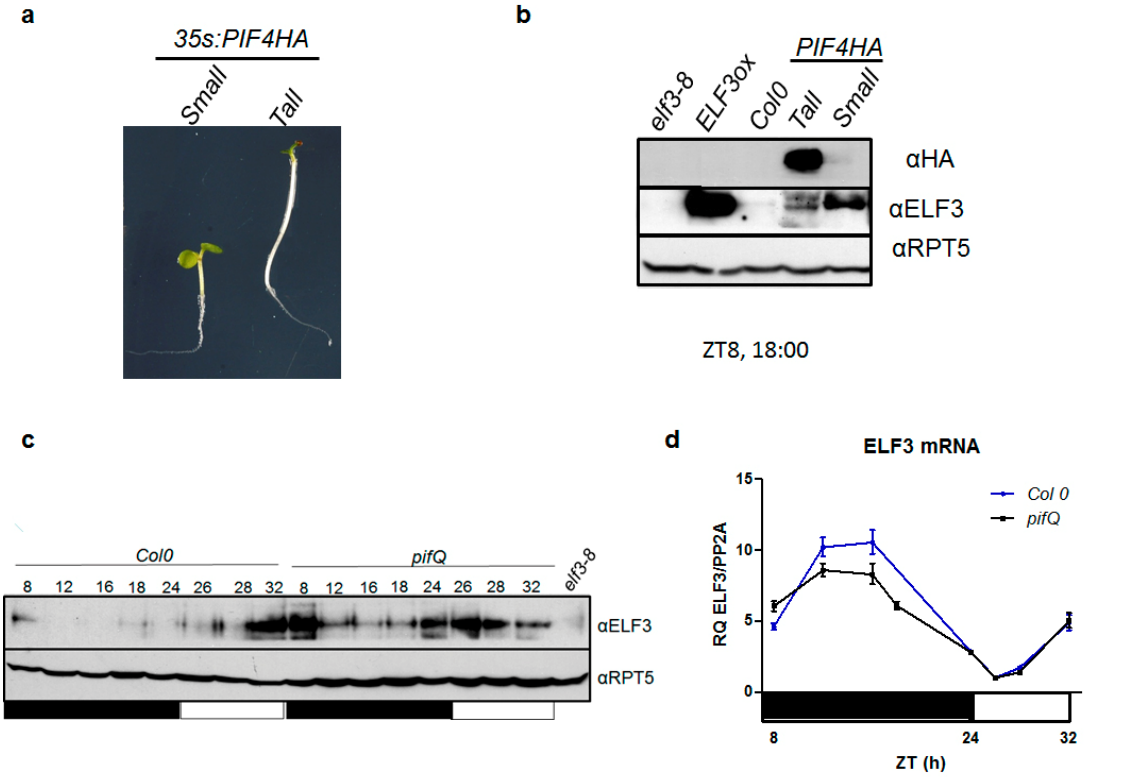

Figure 23. ELF3 protein levels are inversely correlated with levels of expression of the *PIF4* factor. a) Homozygous *35S*::PIF4-HA lines segregate into *tall* and *small* plants due to silencing events. b) The PIF4-HA protein in not detected in *small* seedlings, which accumulate higher levels of the ELF3 protein than the taller (*tall*) plants. c) The photoperiodic profile of ELF3 protein levels is changed in the absence of the PIFs while d) ELF3 expression remains unchanged. Seedlings were grown in short day conditions. Immunodetection with an anti-RPT5 antibody was used as a loading control. qPCR data was normalized to *PP2A* housekeeping gene.

ELF3 has been reported to accumulate during late day to early night (Nusinow, Helfer et al. 2011) and in agreement with this pattern of accumulation of the protein, we observed a peak at ZT8 and 24 hours later at ZT32, in *Col-0* plants. Strikingly, in *pifQ* seedlings, in addition to detect increased levels of the ELF3 protein, this pattern is shifted to a late light/ early morning peak (ZT24 to ZT28), which coincides with the peak of accumulation of the PIF4 protein, observed in transgenic lines expressing this gene under control of its own promoter (Yamashino, Nomoto et al. 2013). Since *pifQ*

seedlings show an identical *ELF3* transcription profile as *Col-0* (Figure 23d), changes in the pattern of the protein denote a regulation at the post-transcriptional level, confirming an inverse correlation of the PIF4 and ELF3 proteins.

Transient activation of *PIF4* decreases ELF3 protein levels.

Since *35S*::PIF4-HA lines are subjected to unintended silencing events, we cannot exclude that higher ELF3 protein levels in these plants, are actually due to silencing of additional genes with a role in ELF3 destabilization. Therefore, we tested the *PIF4* estradiol inducible lines (*XVE*::PIF4) generated in the TRANSPLANTA project, to assess if decreased levels of the ELF3 protein are actually observed after estradiol induction of these plants. Before this, we first verified that application of estradiol activates expression of the *PIF4* transgene and that of the downstream *PIL1* gene target, these lines therefore displaying a proper estradiol regulation of the transgene. For that, plants were grown in MS medium or MS medium supplemented with estradiol, and collected at ZT22, for gene expression analyses (Figure 24). *Col-0* seedlings and transgenic *XVE*::GUS-GFP plants, expressing the GUS-GFP fusion under control of the estradiol inducible promoter, were used as controls, to assess that estradiol treatment did not activate *PIF4* or *PIL1* gene expression. As shown in Figures 24a, b, up-regulated levels of expression of the *PIF4* and *PIL1* transcripts were observed in the *XVE*::PIF4 lines treated with estradiol but not in any of the controls, independently of estradiol application. Therefore, we can conclude that the *XVE*::PIF4 lines express a functional PIF4 protein, these lines thus being suitable to be used in time course experiments.

Hence, these seedlings were grown under SD conditions and treated with estradiol at day 4 after germination. Estradiol was applied at dusk (ZT8), to avoid light destabilization of the newly synthesized protein, and plants were collected at different times during the subsequent dark (ZT36, 40, 44) or light periods (ZT52) for ELF3 protein analyses. As shown in Figure 24d, we were unable to detect any ELF3 protein in the *XVE*::PIF4 (H59) lines treated with estradiol, although a band for this protein was still observed in *Col-0* seedlings treated similarly. Moreover, this band increased in intensity in the sample collected in the light, conditions that are expected to destabilize the endogenous PIF4 protein. Thus, these results, together with those of Figure 23, confirm a role of the PIF4 factor in negatively modulating ELF3 protein levels.

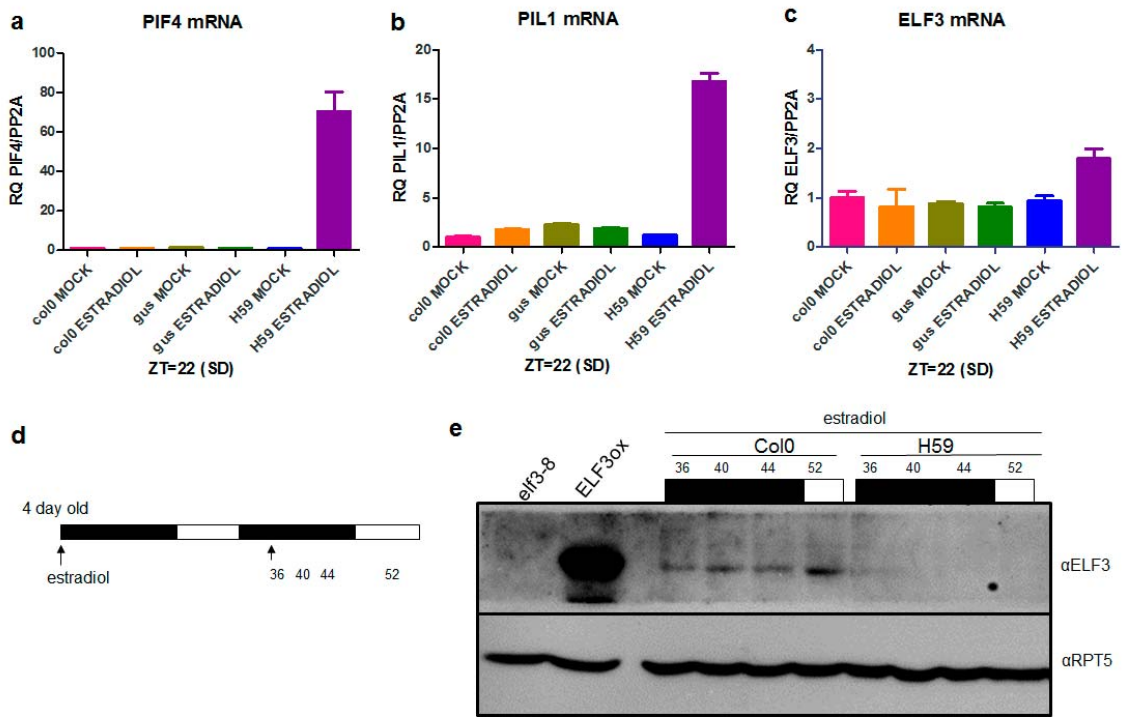

Figure 24. Transient over-expression of the *PIF4* gene leads to reduced ELF3 protein levels. a) Treatment with estradiol induces *PIF4* gene expression in *XVE*:: PIF4 (H59) lines. Activation of this gene is not observed in *Col-0* and *XVE*::GUS-GFP (gus) lines, used as negative controls. b) *PIL1* gene activation in H59 seedlings in response to estradiol. Activation of this gene is observed in the *XVE*::PIF4 lines but not in the controls, hence demonstrating that these lines express a functionally active protein. Data are the means and SD of three technical replicates. c) Transient induction of the PIF4 protein reduces the ELF3 protein down to undetectable levels. Estradiol was applied at dusk (ZT8) to favor dark accumulation of the PIF4 protein. Samples were collected at different times after estradiol application as indicated. Immunodetection with an anti-RPT5 antibody is shown as loading control.

Antagonistic function of ELF3 suppresses the elongated and early flowering phenotypes of *PIF4*ox plants.

The finding that ELF3 represses transcriptional activity of the PIF4 factor, at the same time that PIF4 modulates ELF3 stability, points to a function of these two proteins in a negative feed-back loop preventing excessive growth. *PIF4*ox lines, actually, in addition to be taller than wild-type, show a very early flowering phenotype, undergoing floral transition just after production of 3-4 leaves and completing their life cycle much earlier than the wild-type. Floral stems are also thinner and apical dominance is increased, leading to a reduced yield in seeds. *ELF3*ox lines, in opposite, show a stunted

phenotype and flower later than the wild-type, these plants showing also more leaves of larger size. Floral stems are also thicker and apical dominance is reduced, these plants thus producing more siliques and seeds than control plants. The phenotypes of these over-expresser lines hence indicates that antagonic regulation of these proteins plays an important role in modulating different aspects of plant development in the adult plant. Relevance of this antagonic function, however, cannot be asserted from the analyses of *ELF3oxPIF4ox* lines, since in these plants *PIF4* was expressed under control of its own promoter. *ELF3* over-expression, in fact, besides modulating activity of the PIF4 factor, leads to repression of this transgene, masking functional significance of this protein-protein interaction mechanism. Therefore, we generated lines in which both the *PIF4* and *ELF3* genes were expressed under control of the *35S* promoter, to further demonstrate that antagonic regulation of these proteins plays a relevant role in modulating PIF4 activity. These plants were also used in co-IP studies to further demonstrate interaction of these proteins in stably transformed plants, since these studies were precluded by the fact that the endogenous ELF3 protein is destabilized in *35S*::PIF4-HA lines. *ELF3ox* pollen was used to pollinate *35S*::*PIF4-HA* plants and homozygous *ELF3oxPIF4HA* lines were selected in the F3 offspring. As the parental *35:PIF4-HA* line, these seedlings were subjected to silencing events, segregating into *tall* and *small* plants that were collected separately. As shown in Figure 26g, only the taller seedlings expressed the PIF4-HA construct and these were collected for co-immunoprecipitation assays.

For these studies, five day old seedlings were treated with 50μM MG132, to inhibit proteasomal degradation, and collected in the light. Protein extracts of these plants were immunoprecipitated with anti-HA beads and the purified fraction probed with anti-HA and anti-ELF3 antibodies, to test for the presence of ELF3 in this fraction. As shown in Figure 25, a band corresponding to the pulled-down ELF3 protein was detected in this immunoprecipitated fraction, hence confirming ELF3 and PIF4 interaction in *Arabidopsis* seedlings.

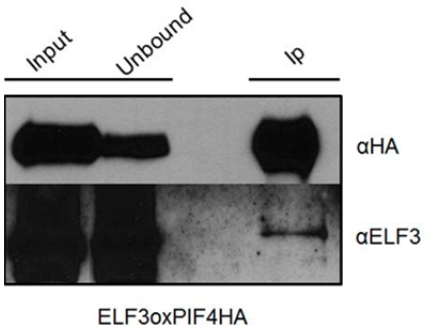

Figure 25. ELF3 and PIF4 interaction in *Arabidopsis* seedlings. The ELF3 protein is pulled-down by immunoprecipitation of the PIF4-HA protein out of protein extracts of ELF3oxPIF4HA over-expression lines. Western blots were probed with anti-HA and anti-ELF3 antibodies. Seedlings were treated with the MG132 proteasome inhibitor to prevent destabilization of these proteins.

.

To analyze the hypocotyl length phenotype of these plants, seedlings were grown under both short-days and cR light conditions and these were measured in 5 days old plants. As show in Figure 26a, b, in SD conditions, hypocotyls of *ELF3*oxPIF4HA plants were actually shorter than the *35S::*PIF4-HA parental line, but differences were smaller than expected for a negative interaction of these two proteins. Silenced *35S::*PIF4-HA seedlings showed similar hypocotyl lengths as *Col-0*, whereas silenced *ELF3*oxPIF4HA plants had similar lengths as the *ELF3ox* lines (Figure 26a,b). Since PIF4 destabilizes the ELF3 protein, SD conditions might not be the conditions of choice to test negative interaction of these proteins, as long nights might promote PIF4 accumulation and lead to complete destabilization of the ELF3 protein. By analyzing *XTR7* gene expression, we actually observed that this transcript accumulates to very high levels in both *35S::*PIF4-HA and *ELF3*oxPIF4HA lines in the night, with repressed expression of this gene only observed in *ELF3*oxPIF4HA seedlings during day time (Figure 26c). This actually indicates that high levels of accumulation of the PIF4 protein in the night lead to complete destabilization of the ELF3 protein, this protein mainly accumulating during day time, when PIF4 is light destabilized. Hence, inhibition of PIF4 transcriptional activity during the day would cause the slight inhibition of hypocotyl growth seen in double over-expressers (Figure 26b). This regulation is exerted at the protein level, as *PIF4* transcript levels are the same in *35s:PIF4-HA* and *ELF3oxPIF4HA* lines (Figure 26d). Likewise, a reduction in *PIL1* and *IAA19* transcript levels is observed in *ELF3oxPIF4HA* lines during the day (Figure 26e,f).

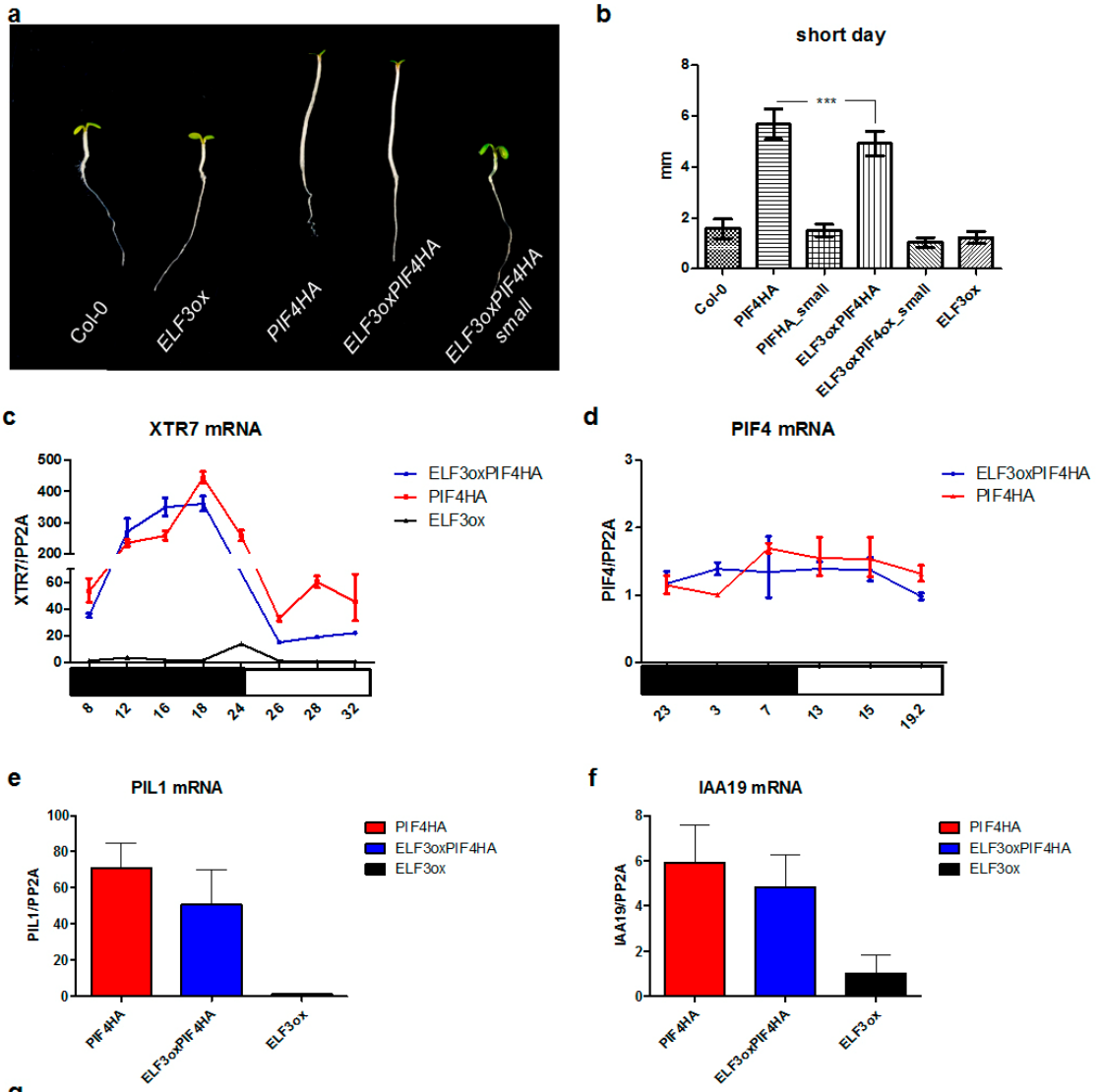

Figure 26. ELF3 inhibits PIF4 activity during the light period in short day conditions. a) Representative plants showing the hypocotyl phenotypes of *35S::*PIF4HA and ELF3oxPIF4HA over-expressers. b) *ELF3*oxPIF4HA seedlings show shorter hypocotyls than the parental PIF4HA line. c) ELF3 over-expression inhibits *XTR7* expression during the light period. d) *PIF4* transcript levels and g) protein PIF4HA levels are comparable between *ELF3*oxPIF4HA and *35S*::PIF4HA. e) *PIL1* and f) *IAA19* expression is also repressed in *ELF3*oxPIF4HA lines in the light g) PIF4 destabilizes the ELF3 protein. b) Data represent the mean and SD of at least 25 seedlings for each genotype. A student t-test shows that differences between PIF4HA and *ELF3*oxPIF4HA seedlings are statistically significant. qPCR values were normalized to the *PP2A* gene. RPT5 was used as a loading control.

Analysis of ELF3 and PIF4 protein levels in the *Tall* (*35S::*PIF4HA*-like)* and *Small* (*ELF3ox-like)* plants confirmed that the PIF4-HA protein is not detected in *Small* seedlings, although ELF3 levels in these plants are comparable to those of *ELF3ox* (Figure 26g). Levels of the ELF3 protein, in addition, were notably decreased in *Tall* seedlings compared to the *Small* silenced ones (Figure 26g). This indicates that ELF3 and PIF4 protein interaction still plays a regulatory role in SD conditions, although PIF4-mediated destabilization of the ELF3 protein supersedes the repressive effects on PIF4 activity of this protein, hence leading to the mild phenotype seen in these plants. Hence, we reasoned that differences in hypocotyl length should still be observed under conditions that favor destabilization of the PIF4 factor, such as continuous light.

As seen in Figure 27a, b, in cR light, hypocotyls of *ELF3*oxPIF4HA lines were actually smaller than those of the PIF4HA parental line, hence confirming a role of ELF3 in repressing PIF4 transcriptional activity in the plant. Continuous red light actually contributed to reduce PIF4 protein levels, enabling stabilization of the ELF3 protein (Figure 27e). *XTR7* gene expression was also more strongly inhibited in *ELF3oxPIF4HA* lines (Figure 27d) consistent with the shorter hypocotyl length of these plants in cR light. Notably, under continuous light conditions ELF3 levels were similar in *ELF3oxPIFHA* and the parental *ELF3ox* line, suggesting that in addition to PIF4, a light repressed component contributes to destabilize this protein in the dark. Actually, the E3 ligase COP1 has been shown to destabilize ELF3 (Yu, Rubio et al. 2008) and light decreases COP1 nuclear abundance (von Arnim and Deng 1994), this E3 ligase being a strong candidate to destabilize ELF3 in the dark.

Remarkably, although at the seedling stage a negative effect of the ELF3 protein on PIF4 transcriptional activity was mainly observed under cR light, *ELF3oxPIF4HA* and *35S*::PIF4-HA lines showed clearly different phenotypes at later developmental stages. As shown in Figure 28a, *ELF3oxPIF4HA* lines have larger leaves at the rosette stage and complete their life cycle later than *35S*::PIF4-HA lines, forming extremely elongated floral stems and setting more seeds than PIF4HA plants. These lines thus display an intermediate phenotype between *35S*::PIF4-HA and *ELF3*ox plants, analyses of *PIL1* gene expression (Figure 28c) in floral stems actually showing that expression of this gene is down-regulated in these plants.

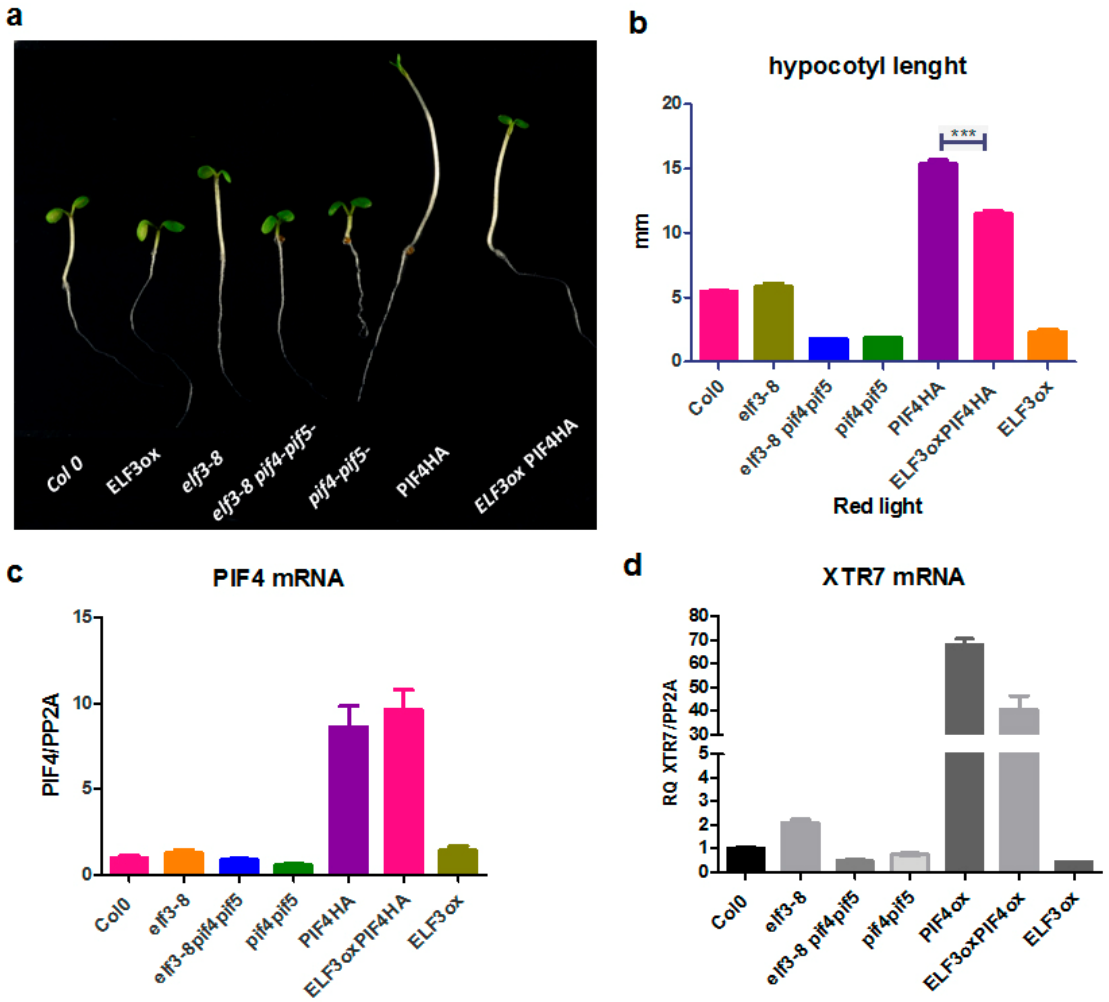

Figure 27. Red light potentiates the negative effect of ELF3 on PIF4 activity. a) Representative plants showing the hypocotyl lengths of the different genotypes under cR light. b) ELF3 represses the hypocotyl length of *35S*::PIF4-HA seedlings. c) *35s:PIF4HA* and *ELF3oxPIF4HA* lines accumulate identical levels of the *PIF4* transcript. d) *XTR7* expression is inhibited by expression of the ELF3 protein. e) ELF3 and PIF4 protein levels remain unchanged cR light, whereas levels of the ELF3 protein are increased in *pif4pif5* and in the silenced PIF4-HA lines. Hypocotyl lengths are the mean and SD of at least 25 seedlings for each genotype. A student t-test shows that differences between lengths of PIF4HA and *ELF3*oxPIF4HA lines are statistically significant. qPCR values were normalized to the *PP2A* gene. RPT5 was used as a loading control.

PIF4 over-expression, in addition, rescued the LD late flowering phenotype of *ELF3*ox plants (Figure 28d). Timing of floral transition is controlled by genes in photoperiodic, autonomous, GA, temperature and vernalization pathways, with cross-talk of these pathways often observed (Fornara, de Montaigu et al. 2010). Interestingly, although PIF4 control of hypocotyl growth and shade response is well established, its role in flowering time control is less understood. PIF4 has been shown to be required for

thermal induced acceleration of floral transition in SDs, and to bind at elevated temperatures to the proximal promoter region of the *FT* gene (Kumar, Lucyshyn et al. 2012). In a recent report, ELF3 has been shown to modulate floral transition by regulating GI stability (Yu, Rubio et al. 2008), by acting as an adaptor protein for COP1 and GI interaction. ELF3 in this way promotes GI destabilization at night, inhibiting *CO* expression and leading to reduced levels of expression of the florigen FT signal. Hence, PIF may promote floral transition by promoting accumulation of GI, thus leading to increased levels of expression of *CO* in the light and *FT* gene activation. Studies to test this possible model of regulation are currently underway.

Discussion and Perspectives.

Altogether, our findings contributed to establish a complex relationship between the ELF3 and PIF4 proteins during early seedling development. These genes were found to control hypocotyl growth via a common regulatory pathway, as we found that the double *pif4pif5* mutation is epistatic to *elf3-8*, suggesting that the PIF4 and PIF5 factors act downstream of ELF3. Analyses of *PIF4* expression in the *elf3-8* mutant actually showed a misregulated pattern of expression of this gene during early night, which leads to high levels of expression of its downstream gene targets. Over-expression of the ELF3 protein in *PIF4*ox lines, in addition, repressed the elongated phenotype of these plants although in subsequent analyses we observed that this effect is in part mediated by inhibition of *PIF4* expression, due to transcriptional repression of the native promoter driving expression of this gene. Such an effect agreed with the finding that ELF3 interacts with the ELF4 and LUX/NOX proteins to form the so called “Evening Complex” (EC), repressing morning-phased genes at dusk (Nusinow, Helfer et al. 2011). This repressive complex actually binds via the LUX transcription factor the promoters of *PIF4/5*, to modulate circadian expression of these genes and rhythmic hypocotyl growth. However, *elf3-8PIF4ox* plants display longer hypocotyls and this additive effect is independent of *PIF4* transcription, hence suggesting and additional regulation of these proteins at the post-transcriptional level.

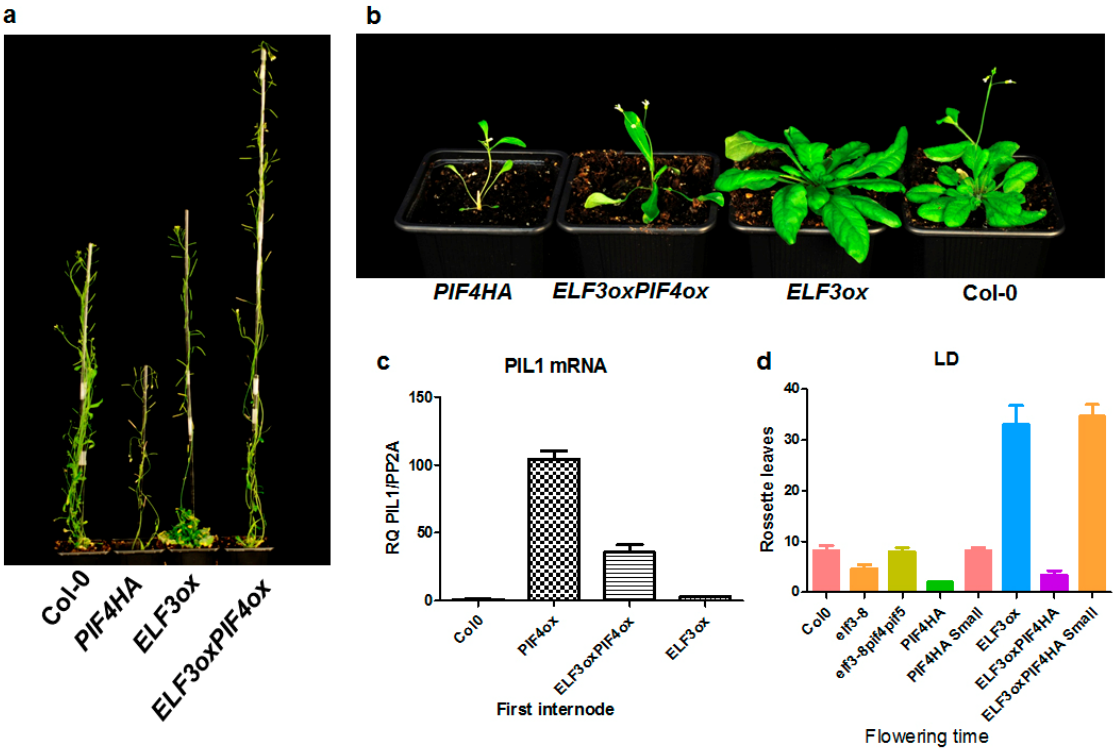

Figure 28. PIF4-ELF3 interaction modulates plant development. a) ELF3oxPIF4HA lines complete their life cycle later than *35S*::PIF4-HA lines and display extremely elongated floral stems. b) The size of the leaves is also increased, hence showing an intermediate phenotype between *35S*::PIF4-HA and *ELF3*ox plants. c) PIL1 expression is decreased in the first internode of the inflorescence stem. d) PIF4 overexpression rescues the late flowering phenotype of ELF3 under long day conditions. qPCR values were normalized to the *PP2A* gene. For flowering time, the mean and SD of the number of rosette leaves at floral transition is represented. Number of leaves was measured in at least 10 plants for each genotype

We actually found that these proteins are able to directly interact, by using yeast two hybrid, and BiFC assays, and by co-IP of these proteins out of *N benthamiana* leaves and *Arabidopsis* plants. By mapping the interacting domains of these proteins, we could establish that the C-terminal domain of ELF3 binds the bHLH domain of the PIF4 factor. This domain is highly conserved among all PIF family members, ELF3 actually interacting with other members of the gene family, such as PIF1, HFR1 and SPT. Since this conserved domain is implicated in dimerization and DNA recognition, these results point to a role of ELF3 in the inhibition of PIF4 transcriptional activity, as seen for the DELLAs, HFR1 and more recently PHYB (de Lucas, Daviere et al. 2008; Hornitschek, Lorrain et al. 2009; Hao, Oh et al. 2012; Park, Park et al. 2012). In transient trans-

activation assays we have actually observed that ELF3 represses PIF4 transcriptional activity in a similar manner as the RGA repressor. This inhibitory effect is also observed in *Arabidopsis* plants although it is difficult to separate this effect from the transcriptional control as a component of the EC complex. We have seen that PIF4 retains transcriptional activity under continuous light, thought to destabilize this factor, and established that in these conditions this post-transcriptional regulatory mechanism plays an important role. Actually, PIF4 is widely accepted to be unstable in the light due to PHYB-directed phosphorylation to mark this protein for proteasomal degradation (Nozue, Covington et al. 2007). Studies of stability of this factor have been conducted in plants expressing this protein under control of a constitutive promoter, and recently, we and others (Yamashino, Nomoto et al. 2013) observed that when expressed under control of its own promoter, the protein accumulates in the light. The PIF4-HA protein in transgenic p*PIF4*::PIF4-HA lines actually follows a similar profile as the transcript, accumulating by the end of the night to peak during the day and be reduced early in the night. Notably, expression of the *XTR7* and *PIL1* gene targets peaks at late night, indicating that PIF4 transcriptional activity is repressed during the light period. Our findings that *PIF4* expression levels barely change in *ELF3*ox lines in cR, but downstream gene expression is repressed, also after a switch to FR light, further demonstrates the biological relevance of this protein-protein interaction mechanism. This was further confirmed by generating *ELF3oxPIF4HA* lines, where expression of these genes is driven by the constitutive *35S* promoter. These lines, in cR, show shorter hypocotyls than the parental *35S:*PIF4HA line and reduced levels of expression of the *XTR7* gene, hence identifying ELF3 as a novel negative regulator of PIF4 transcriptional activity. Strikingly, inhibitory effects of this protein were much smaller in SD photoperiodic conditions, analyses of *XTR7* gene expression showing that this gene is repressed during the day but not in the night. This supports the idea that ELF3 plays an important role in inhibiting PIF4 activity in the light, in which ELF3 is stabilized. Interestingly, we observed that ELF3 protein levels are also regulated by PIFs, with this protein found to accumulate to higher levels in the *pifQ* mutant and to be unstable in PIF4ox lines. In these lines, ELF3 levels correlate with levels of the PHYB photoreceptor and thus we cannot exclude a role of PHYB in stabilizing this protein, as PHYB interacts with the N-terminal domain of the ELF3 protein. These findings suggest the PIFs not only function as outputs of the clock but may also modulate clock function, by regulating levels of the ELF3 feed-back loop protein. Also, although ELF3 repressive

effects on PIF4 activity were mild at the seedling level, older *ELF3oxPIF4HA* plants showed in many respects as intermediate phenotype between *ELF3*ox and *35S*::PIF4-HA plants, indicating that this interaction is more relevant in adult plants. *ELF3*oxPIF4HA plants actually displayed larger leaves than *35S*::PIF4HA lines and rescued the late flowering phenotype of *ELF3*ox plants thus leading to robust plants that mimic ELF3 over-expressers without an associated delay in floral transition.

Chapter II: ELF3-DELLA interaction modulates GA feed-back regulation and defines the organ-specific pattern of accumulation of DELLAs .

Introduction.

In a two-hybrid screen with the RGA bait protein, we identified ELF3 as one of the interacting partners for these repressors. Since previous work in the lab had shown that DELLAs bind the PIFs bHLH domain and sequester these factors in an inactive form unable to bind to DNA, we hypothesized that further interaction with ELF3 may contribute to diurnally regulate formation of the DELLA-PIFs complex, hence linking GA signaling with the clock. ELF3 binds the ELF4 and LUX factors to form the evening complex (EC), a negative regulatory complex that represses *PIF4* expression at dusk (Nusinow, Helfer et al. 2011; Herrero, Kolmos et al. 2012). Transcription of this factor is induced at late night and peaks during the day, with PHYB-mediated destabilization repressing PIF4 activity in the light. Hence, combination of EC and PHYB regulation shapes rhythmic elongation of hypocotyl growth, by restricting PIF4 activity at the end of the night. Results reported in Chapter I, in addition, show that ELF3 represses PIF4 transcriptional activity by directly interacting with this factor, suggesting a role for ELF3-PIF4 interaction in gating the hypocotyl elongation response limiting PIF4 activity during the light period. The finding that ELF3 interacts with the DELLAs adds an additional layer of regulation to the diurnal control of PIF4 activity, either by favoring formation of a ternary ELF3-PIF4-DELLA complex that increases PIF4-DELLA binding affinity or by playing an independent role in diurnal regulation of DELLA levels. DELLAs were in fact shown to accumulate in the light and to be destabilized in darkness (Achard, Liao et al. 2007), being postulated that increased GA biosynthesis in the dark mediates degradation of these repressors during the night. However, studies to demonstrate that this destabilization depends only on GA levels have not been carried out. To gain evidence on the possible role of this interaction we set to map the interacting domains in both proteins and to analyze if RGA levels are modified in *ELF3*ox and *elf3-8* lines.

ELF3 interacts with the DELLA proteins.

Yeast two hybrid screens that led to the identification of ELF3 as a DELLA interacting partner were conducted with the *St*GAI potato protein. A fragment of the protein lacking the DELLA domain (M5-GAI, Figure 29a) was used in these assays as this fragment did not show the problems of auto-activation seen for the full-length protein. Four

independent partial clones for the *St*ELF3 protein could be identified in the screen (Rodriguez-Falcon 2003). While in potato DELLAs are encoded by a single gene, five different genes for these repressors have been identified in *Arabidopsis* (Tyler, Thomas et al. 2004; Gallego-Bartolome, Alabadi et al. 2011), with different functions of these genes relying in their different expression patterns (Gallego-Bartolome, Minguet et al. 2010). It has also been reported that different DELLAs show distinct affinities to their interaction partners (Suzuki, Park et al. 2009). Thus, to test if ELF3 interacts with these five DELLA proteins, equivalent M5 deletions (de Lucas, Daviere et al. 2008) were generated for each of these *Arabidopsis* repressors and used in yeast assays with the ELF3 prey protein. As shown in Figure 29b, all five DELLA were able to interact with AtELF3, hence pointing to a functional relationship of these proteins. Deletions of the GAI protein described in (de Lucas, Daviere et al. 2008) were also used to define which domain of this repressor mediates interaction with ELF3. Two different fragments were generated by *Eco*RI digestion of the gene, which cuts within the nucleotide sequence encoding the second leucine heptad repeat, leading to an N-terminal fragment (F1, 1 to 362 aa) comprising the DELLA, LHI and VHIID regions and a C-terminal fragment (Relig, 368 to 588 aa) comprising the SH2 domain (Figure 29a). Deletions del1, comprising from the VHIID region to the C-terminal end, and del2, from the second leucine heptad repeat (LHII) to the C-terminal end of the protein, were also used in these assays. These conserved domains are also shared by other members of the GRAS family of proteins (Pysh, Wysocka-Diller et al. 1999). As seen in Figure 29c, ELF3 binds the M5 and F1 fragments of the GAI protein, but does not interact with the del1 fragment, hence indicating that the leucine heptad repeat (LHI) region mediates ELF3 interaction. Interestingly, this same domain was also found to interact with PIF4 (de Lucas, Daviere et al. 2008), hence excluding formation of a GAI-ELF3-PIF4 ternary complex via interaction with the GAI protein. To map the interacting domain in the ELF3 protein, deletions that were generated to map ELF3-PIF4 interaction, were also used here. As shown in Figure 29d, the M-ELF3 domain comprising the conserved central region of the ELF3 protein shows a strong interaction with the M5-GAI protein, this domain therefore binding the DELLAs. Unfortunately, the N-ELF3 domain showed auto-activation when fused to the GAL4-AD domain and could not be assessed in these interaction assays.

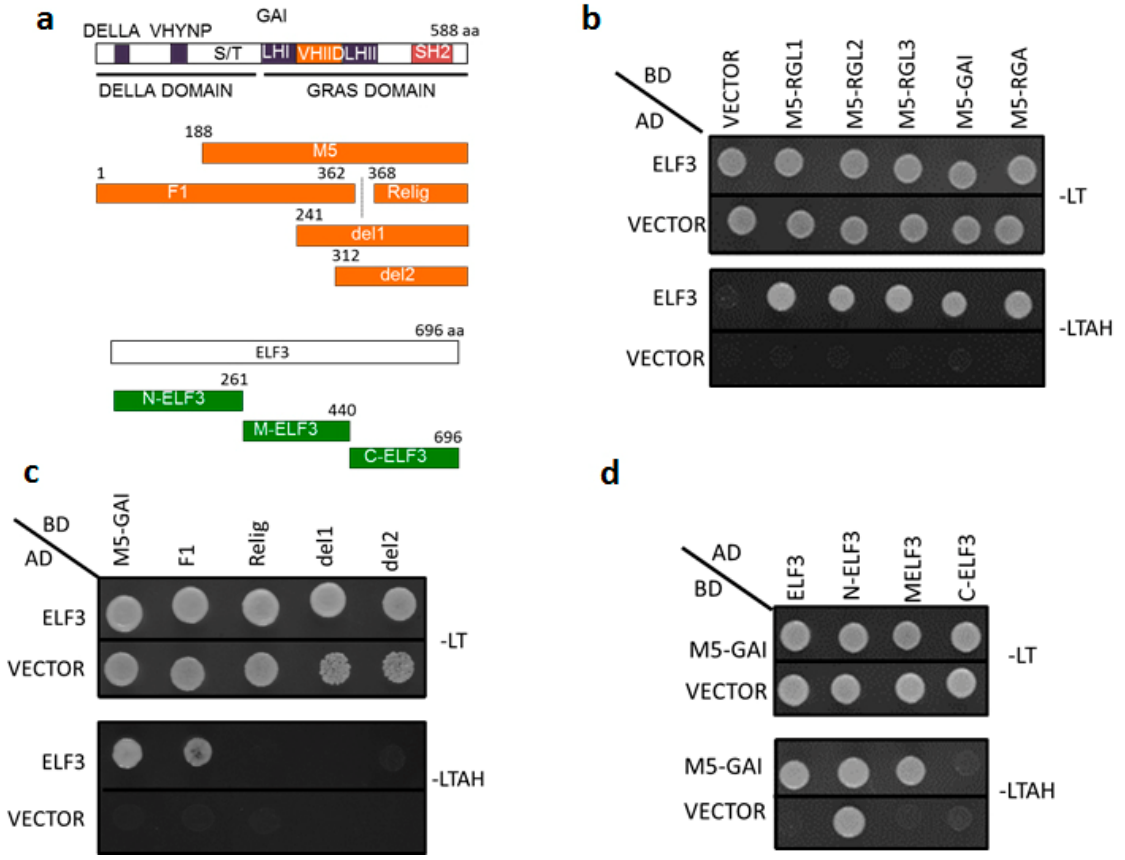

Figure 29. DELLA proteins interact with ELF3 in Y2H. a) Deletions used for the interaction assays. b) ELF3 interacts with all five *Arabidopsis* DELLAs. c) The fragments M5 and F1 containing the first leucine heptad (LHI) repeat show a positive interaction, while del1 lacking this motif does no longer interact, suggesting that the LHI domain mediates ELF3 interaction. d) The ELF3 central domain (M-ELF3) is sufficient for interaction with the GAI protein. The N-ELF3 shows auto-activation when fused to the GAL4 activation domain (AD).

The conserved M-ELF3 domain is shared by other DELLA interacting proteins.

ELF3 is characterized by three highly conserved domains (N-ELF3, M-ELF3 and C-ELF3), which are present in all ELF3 plant orthologues (Liu, Covington et al. 2001). The N-ELF3 domain mediates interaction with the PHYB and COP1 proteins, while M-ELF3 has been shown to bind the ELF4 protein (Nusinow, Helfer et al. 2011; Herrero, Kolmos et al. 2012), and now we showed that this domain is also involved in DELLA interaction. Interestingly, when this region is aligned with the uniprot protein database, using the HMMer and HHpred (based in hidden profile Markow models) programs, highly related domains could also identified in other *Arabidopsis* proteins (Figure 30a).

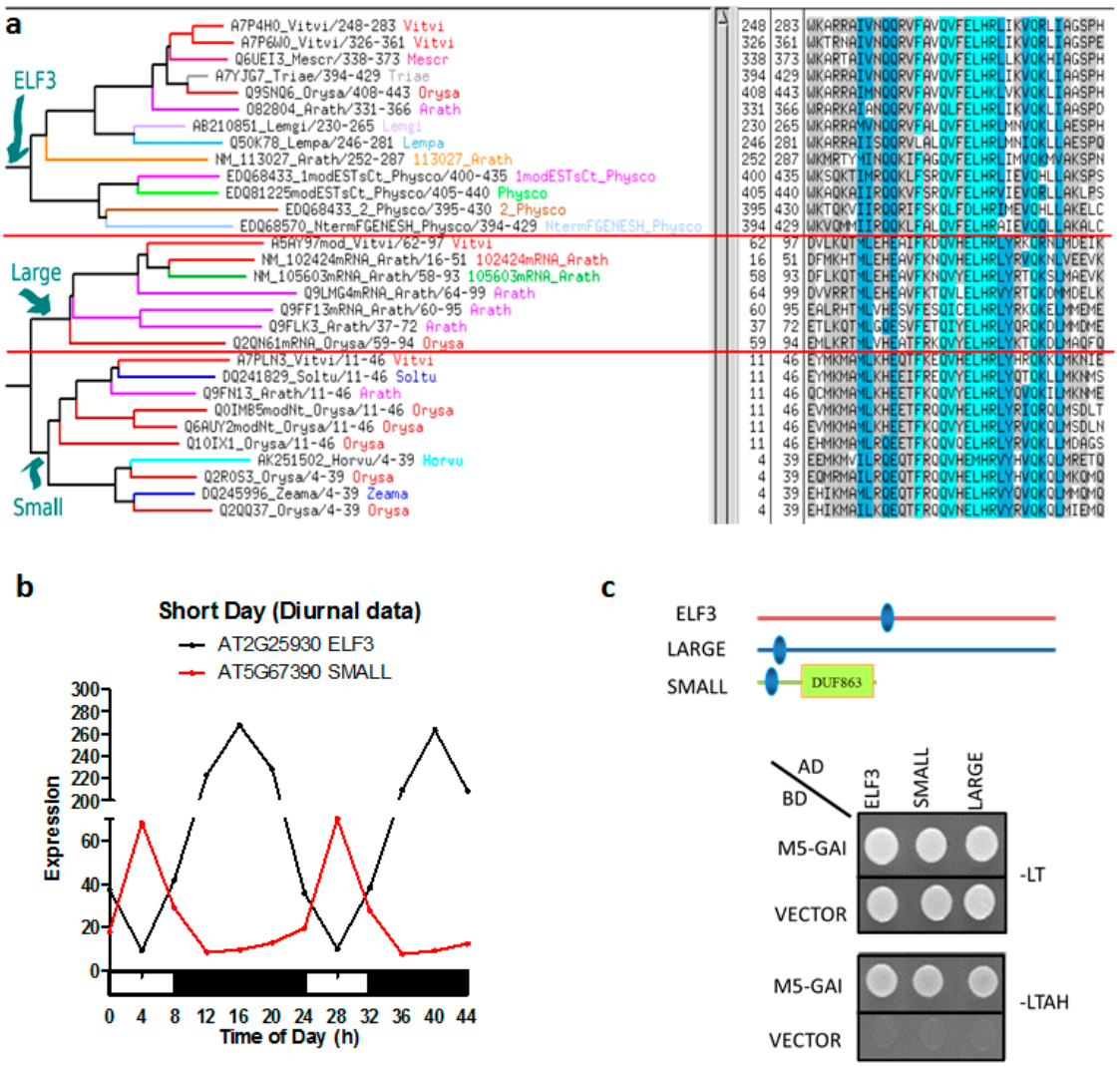

Figure 30. The conserved M-ELF3 domain is a new motif for interaction with the DELLAs. a) Uniprot database proteins found to share the M-ELF3 central domain. b) Diurnal data showing an antiphasic expression of the ELF3 and At5g67390 (SMALL) transcripts. c) The conserved M-ELF3 like motif of the LARGE and SMALL proteins interacts with M5-RGA in yeast cells.

To test if all these domains bind the DELLAs, we selected two proteins (Q9FN13 and Q9FF13) representative of each of these two additional homology clades and amplified these regions, to test them for interaction with the DELLAs (Figure 30c). Q9FN13 (named SMALL) corresponds to the At5g67390 gene, encoding a 176 aa protein of unknown function, with a conserved DUF863 domain. Although this gene has not been characterized at the protein level, its transcript is ubiquitously expressed and quite remarkably, shows an antiphasic profile of expression with ELF3 (Figure 30b), which suggests that this gene is clock regulated. The second of these proteins, Q9FF13 (named LARGE), corresponds to the gene At5g07790, for which evidence has not

been obtained at the transcript level. As shown in Figure 30c, both amplified fragments were able to interact with the M5-GAI protein, thus confirming a function of this conserved domain as a novel DELLA interacting domain. We did not analyze further these proteins, but the observation that they interact with the DELLAs might be useful to assign a function to these genes.

ELF3 binds the DELLA proteins *in vivo*.

Interaction of the *Arabidopsis* ELF3 and RGA proteins was confirmed *in planta* by transiently expressing both proteins in *N. benthamiana* leaves. For RGA expression, we used a construct expressing the RGA protein fused to GST, under control of the *35S* promoter (*35S::*RGA-GST). This construct was co-expressed with the *35S::*ELF3-CFP construct described in Figure 15. As shown in Figure 31a, a band corresponding to RGA-GST protein was pulled-down after immunoprecipitation of the ELF3-CFP protein with anti-GFP agarose beads (Figure 31a). This interaction was also confirmed by bi-molecular fluorescence complementation assays (BiFC), where the nYFC-RGA and cYFC-ELF3 constructs in the YFN43 and YFC43 vectors, were co-infiltrated in *N. benthamiana* leaves. A nuclear fluorescence signal corresponding to YFP was detected in leaves co-expressing both constructs, but not in leaves expressing the RGA or ELF3 constructs and the complementary empty vector, used as negative controls (Figure 31b).

We can conclude from these studies that ELF3 interacts with all five DELLAs and that this interaction is mediated by the LHI repeat region of the DELLAs and the M-ELF3 region of the ELF3 protein. Other proteins share an M-ELF3 related domain and were found to bind the GAI repressor, hence identifying this domain as a novel DELLA interacting domain. Interaction of the ELF3 and RGA *Arabidopsis* proteins was also confirmed *in vivo*, hence prompting us to assess the possible functional implications of this interaction.

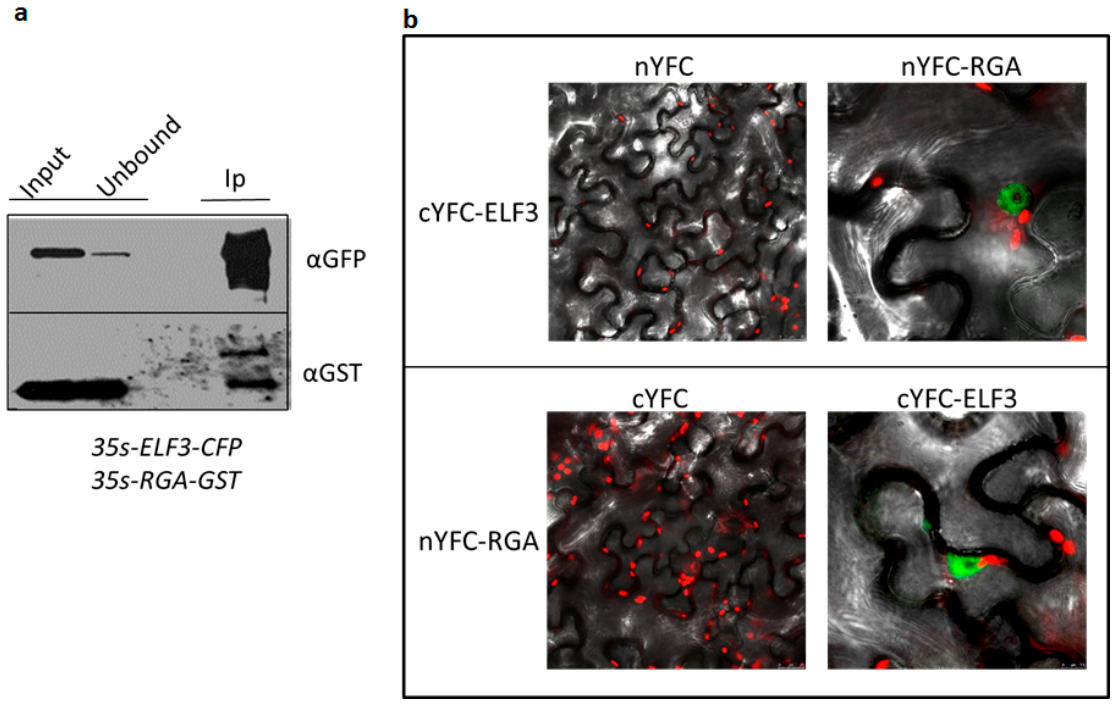

Figure 31. ELF3 interacts with RGA in plant cells. a) RGA-GST is pulled-down after immunoprecipitation of the ELF3-CFP protein out of *benthamiana* leaf extracts co-expressing both proteins. b) Nuclear YFP fluorescence is observed in leaves expressing the nYFC-RGA and cYFC-ELF3 constructs, but not in control leaves infiltrated with either of these constructs and the complementary BiFC empty

RGA levels are elevated in the *elf3-8* and *cop1-4* mutants.

The biochemical function of ELF3 is to the date not fully understood. Several lines of evidence point to a function of this protein as a nuclear scaffold for other proteins interaction (Yu, Rubio et al. 2008; Dixon, Knox et al. 2011; Nusinow, Helfer et al. 2011; Chow, Helfer et al. 2012; Herrero, Kolmos et al. 2012). In particular, ELF3 has been shown to serve as a substrate adaptor for the E3 ligase COP1 (binds the N-ELF3 domain) and the clock and flowering time protein GI, hence triggering GI degradation and repressing floral transition in SD (Yu, Rubio et al. 2008). Interestingly, GAI, like GI, interacts with the M-ELF3 domain, which suggests that ELF3 may play a role in modulating DELLA's stability. To test this hypothesis, we set out to analyze RGA protein levels in the different ELF3 and COP1 backgrounds, using an anti-RGA specific antibody, kindly provided by Claus Schwechheimer.

Noteworthy, RGA has been reported to accumulate in the nucleus in the light, but to be destabilized in darkness (Achard, Liao et al. 2007). COP1, by contrary, accumulates in the dark, but is depleted from the nucleus in the light. Activated CRY1 and CRY2 repress COP1 activity by binding the COP1 protein and competing for complex

formation with SPA1 (von Arnim and Deng 1994; Wei, Chamovitz et al. 1994; von Arnim, Osterlund et al. 1997; Ang, Chattopadhyay et al. 1998; Yu, Rubio et al. 2008; Jang, Henriques et al. 2010). COP1, however, is not totally inactive in the light, as it stills targets some substrates for degradation during the day. Changes in ELF3 protein levels in the *cop1-4* mutant background, for instance, are mostly seen at dusk (ZT8), suggesting that COP1 destabilizes the ELF3 protein during this interval of the day (Yu, Rubio et al. 2008). Therefore, we set out to analyze hypocotyl lengths and levels of the RGA repressor in the *cop1-4* mutant and in double *cop1-4elf3-8* and *cop1-4ELF3*ox plants.

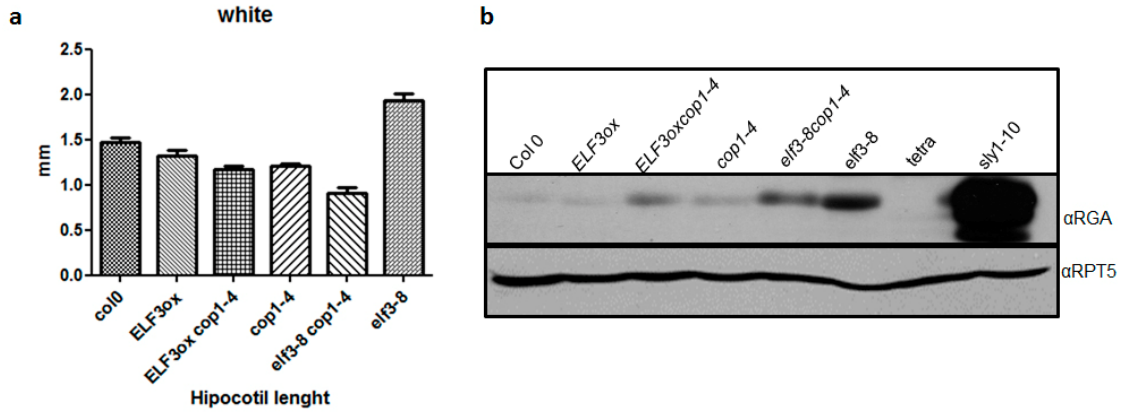

Figure 32. Hypocotyl length and RGA protein levels are regulated by ELF3 and the E3 ligase COP1. a) *ELF3ox* plants and mutants carrying the *cop1-4* allele are smaller than the wild-type, while *elf3-8* is taller than *Col-0*. The *cop1-4* mutation is epistatic to *elf3-8* for hypocotyl growth. b) *RGA* protein levels are increased in *cop1-4* seedlings and surprisingly are increased to even higher levels in the *elf3-8* mutant. Seedlings were grown under SD conditions and harvested during the day.

As seen in Figure 32a, hypocotyls of *cop1-4* mutants are shorter than the *Col-0* wild-type as already reported (Hicks, Albertson et al. 2001). *elf3-8* mutants, in turn, have tall hypocotyls, while *ELF3*ox plants are shorter than wild-type, as seen in Chapter I. Interestingly, the *cop1-4* mutation is epistatic to *elf3-8* for hypocotyl growth but not for flowering, as *cop1-4elf3-8* seedlings show short hypocotyls but flower as early as *elf3-8* mutant plants (Yu, Rubio et al. 2008). Levels of the RGA protein were found to be increased in the *cop1-4* mutant background, but surprisingly levels of this repressor were increased to an even greater extent in the *elf3-8* genotypes (Figure 32b). Actually, *elf3-8* seedlings display the highest levels of accumulation of the RGA repressor, although hypocotyls of these seedlings are elongated, thus evidencing that ELF3 is required for destabilization of DELLAs and that, in the absence of ELF3, the DELLAs are unable to restrain hypocotyl growth. Hence, although the de-etiolated phenotype of

cop1-4 seedlings had been explained by an accumulation of HY5 (Lau and Deng 2010), these observations would point to a further contribution of the DELLAs to this phenotype.

To further define the effects of the COP1 and ELF3 proteins on the levels of accumulation of RGA, we analyzed the levels of this repressor in seedlings shifted from dark to light conditions, and in seedlings shifted from light to dark. DELLAs were actually shown to promote photomorphogenesis by accumulating in the nucleus upon transfer from dark to light conditions, whereas they are destabilized when seedlings are shifted from light to dark (Achard, Liao et al. 2007). Therefore, we decided to analyze levels of the RGA protein in *elf3-8* and *cop1-6* seedlings, carrying a weaker allele than the *cop1-4* mutation, after the switch from light to dark or from dark to light conditions. As shown in Figure 32a, RGA levels were found to be reduced upon transfer to dark conditions, in wild-type *Col-0* seedlings grown in continuous white light. Actually, a rapid destabilization of the protein is observed after the shift to dark conditions, with protein levels being again partially restored after 24 hours in the dark. Noteworthy, a similar destabilization pattern was observed in *cop1-6* mutant seedlings, although protein levels were much higher in this mutant background. *elf3-8* seedlings, by contrary, did not show a decrease in RGA levels upon transfer to the dark, hence pointing to a role of ELF3 in DELLAs destabilization during light to dark transition (Figure 33a). Interestingly, the pattern of RGA accumulation was strikingly different in seedlings transferred from darkness to white light (Figure 33b). While in *Col-0* seedlings RGA accumulates after the shift to light, levels of the RGA repressor did not change in the *elf3-8* mutant background, and this protein was found to be destabilized in *cop1-6* seedlings. In dark-grown *cop1-6* seedlings, RGA levels are actually lower than in the *Col-0* wild-type, and this protein not only does not accumulate, but was further destabilized in the light. Thus, whereas RGA levels are elevated in *elf3-8* seedlings independently of light conditions, in *cop1-6* mutants this repressor protein accumulates in the light and in seedlings transferred from the light to darkness, but is reduced in dark-grown seedlings to be further destabilized upon transfer to the light. These findings thus point to an important role of the COP1 and ELF3 proteins in regulating RGA levels, with ELF3 being required for the changes in RGA stability observed in response to light or dark transitions, while COP1 would exert opposite regulatory functions in seedlings grown in continuous light compared to those grown in darkness, as evidenced by the increased levels of the RGA protein observed in light grown *cop1-6* seedlings and reduced levels of this repressor in seedlings grown in the dark.

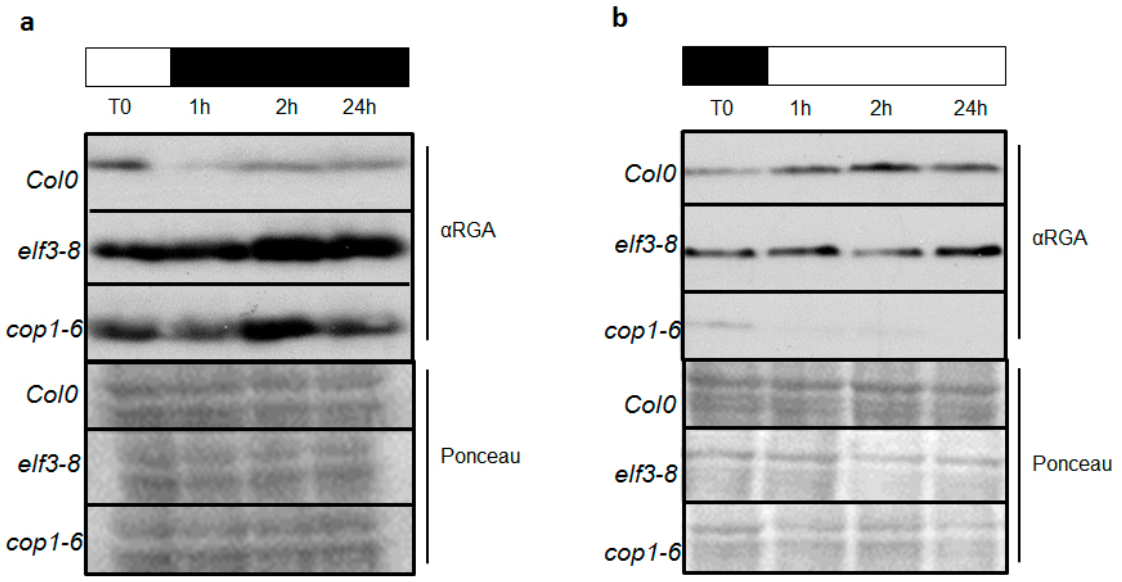

Figure 33. *cop1-6* and *elf3-8* seedlings show impaired RGA protein levels in response to a switch to light or dark conditions. a) RGA protein levels are reduced in *Col-0* and *cop1-6* seedlings upon transfer from light to dark conditions. A similar reduction in RGA protein levels is not observed in *elf3-8* seedlings. b) RGA accumulates in wild-type *Col-0* seedlings upon transfer from dark to light conditions. A similar stabilization of the protein is not observed in *elf3-8* seedlings. Strikingly, *cop1-6* seedlings grown in darkness show reduced levels of RGA and this protein is further destabilized when seedlings are shifted to the light.

As the different response of *elf3-8* and *cop1-6* mutants to the switch from dark to light or light to dark conditions seemed to evidence two independent mechanisms of regulation of RGA protein stability, we decided to analyze levels of this repressor under simulated shade conditions, which were reported to lead to RGA destabilization. Actually, RGA has been reported to function as a negative regulator of the shade avoidance syndrome (SAS), and to be destabilized in FR-enriched light simulating the light filtrated through a canopy (Djakovic-Petrovic, de Wit et al. 2007). COP1 plays a prevalent role in this response, since *cop1* mutants do not elongate in the shade (McNellis, von Arnim et al. 1994; Roig-Villanova, Bou et al. 2006; Crocco, Holm et al. 2010) and COP1 re-accumulates in the nucleus under FR-enriched light (Pacin, Legris et al. 2013). Allelic variation in the ELF3 protein has also been reported to underline the differential shade response phenotype of the *Bay* and *Sha* ecotypes (Jimenez-Gomez, Wallace et al. 2010; Coluccio, Sanchez et al. 2011). To this respect, ELF3 and RGA function as negative regulators of the SAS response, whereas COP1 promotes SAS. Therefore, we considered that analyzing RGA protein levels in the *elf3-8* and *cop1-6* backgrounds, under SAS conditions, might provide relevant information on the

mechanism of action of both ELF3 and COP1 proteins and help to establish if they modulate RGA protein levels via a common signaling pathway. SAS is triggered in response to a change from high to low R/FR ratio in the incident light, which modifies the number of Pfr activated molecules of PHYB (reviewed in (Franklin 2008). RGA and ELF3 were reported to have a major effect in hypocotyl growth under red light (Liu, Covington et al. 2001; Kim, Hicks et al. 2005; de Lucas, Daviere et al. 2008). RGA accumulates in red light and *phyb* mutants show a delayed light-dependent accumulation of this repressor (Achard, Liao et al. 2007). ELF3, on the other hand, is also stabilized in red light (Figure 20) and was shown to directly interact with PHYB (Liu, Covington et al. 2001). Even functional relevance of this interaction is not fully understood, several lines of evidence support a role of PHYB in modulating ELF3 activity in the light (Herrero-Soriano 2011; Kolmos, Herrero et al. 2011). Interestingly, both PHYB (Liu, Covington et al. 2001) and COP1 (Yu, Rubio et al. 2008) proteins bind the N-terminal domain of ELF3, suggesting that both proteins probably compete for ELF3 interaction. Hence, shade avoidance conditions due to reduce nuclear levels of the PHYB photoreceptor, are expected to favor interaction of the ELF3 protein with COP1, providing an appropriate frame to analyze the role of the COP1-ELF3 complex in regulation of RGA protein levels. Therefore, seedlings were grown under continuous red light, to favor accumulation of the RGA and PHYB proteins, and were then subjected to a 15 min pulse of FR light before transfer to dark conditions, to promote COP1 activity (Figure 34a). This end of the day (EOD) FR pulse in fact represses PHYB activity, mimics shade conditions and promotes hypocotyl elongation (Sellaro, Pacin et al. 2012), whereas prolonged FR treatments were reported to inhibit COP1 activity (McNellis, von Arnim et al. 1994).

As shown in Figure 34c, in cR light that activates PHYB signaling, RGA accumulates in all genetic backgrounds. A 15 min FR pulse, before transfer to dark conditions, inactivates PHYB and destabilizes the RGA repressor in wild-type *Col-0* and *ELF3*ox seedlings. A reduction in RGA protein levels, however, is not observed in the *cop1-4* and *elf3-8* mutant backgrounds, hence pointing to a role of both ELF3 and COP1 proteins in RGA destabilization in the dark. As seen in Figure 34b, ELF3 protein levels were not affected by these light changes, as a band of similar intensity is observed in *ELF3*ox seedlings under cR or 1h and 4h of FR pulse + darkness (note that these result are different from those in Figure 21, where ELF3 was found to be destabilized by a prolonged FR treatment).

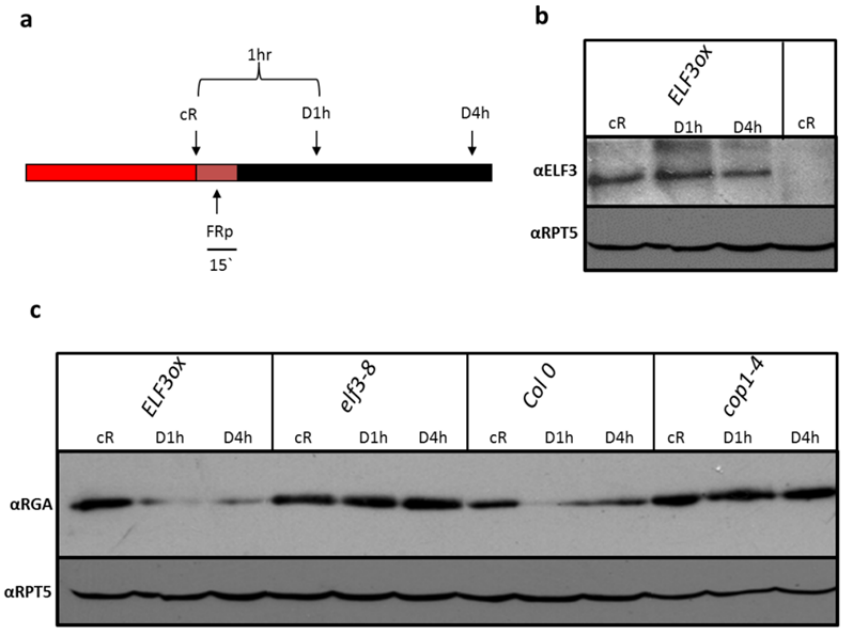

Figure 34. COP1 and ELF3-dependent destabilization of the RGA repressor is modulated by phytochrome B. a) Light conditions used to grow the plants. Seedlings were grown under cR light that promotes RGA and ELF3 accumulation and the nuclear translocation of the Pfr active form of PHYB. They were then given a 15 min pulse of FR light, to convert PHYB into its inactive Pfr form and then transferred to dark conditions to further promote COP1 activity. Samples were collected in cR and 1h and 4h after the FR pulse and dark treatment. b) A short (15 min) pulse of FR light does not affect stability of the ELF3 protein in *ELF3*ox lines. c) RGA is destabilized in *Col-0* and *ELF3*ox lines after PHYB inactivation and transfer to dark conditions. A similar destabilization is not observed in the *elf3-8* and *cop1-4* mutant plants.

These observations are consistent with our previous findings showing that RGA accumulates in white light in the *elf3-8* and *cop1-4* mutants (Figure 31b), hence supporting a role of the COP1 and ELF3 proteins in DELLAs destabilization and showing that PHYB activation suppresses activity of these proteins. Actually, although ELF3 was found to interact *in vitro* with both Pr and Pfr forms of PHYB (Liu, Covington et al. 2001), only the active Pfr form is translocated into the nucleus (Rausenberger, Hussong et al. 2010). A short FR pulse leads to the conversion of PHYB Pfr back to its inactive Pr form, this form being excluded from the nucleus and nuclear accumulation of COP1 further promoted in darkness (von Arnim, Osterlund et al. 1997), thus leading to RGA destabilization by favoring COP1-ELF3 interaction. These findings suggest an environment dependency in which either an ELF3-DELLA or ELF3-COP1-DELLA regulation could occur. To further verify this, RGA levels were analyzed in the *elf3-8* and *ELF3ox* in diurnal conditions. Also, since DELLAs mediate feed-back regulation of

GA synthesis, we analyzed diurnal expression of the GA biosynthetic and catabolic enzymes in the ELF3 lines.

ELF3 modulates RGA stability.

ELF3 is known to function as a negative feed-back loop of the clock, activity of this protein being required for rhythmic hypocotyl growth since it recruits the ELF4 and LUX proteins to the evening repressor complex (EC), shown to repress *PIF4*/*PIF5* gene expression at dusk (Nusinow, Helfer et al. 2011; Herrero, Kolmos et al. 2012). Additionally, results reported in Chapter I, demonstrate that this protein is also implicated in regulating transcriptional activity of the PIF4 and PIF5 factors, hence contributing to fine-tune regulate PIFs activity. Noteworthy, diurnal oscillation of GA signaling has also been proposed to contribute to regulate rhythmic growth, with diurnal expression of the GA GID1-receptor playing a central role in circadian regulation of GA sensitivity and in DELLAs accumulation during daytime (Arana, Marin-de la Rosa et al. 2011). Constitutive GID1 expression actually expands the daily growth period, while loss of DELLA function, in the quintuple *della* mutant, leads to continuous arrhythmic growth (Arana, Marin-de la Rosa et al. 2011; Marin-de la Rosa, Alabadi et al. 2011). Most GA biosynthetic and signaling genes, in addition, exhibit a circadian pattern of expression (Hisamatsu, King et al. 2005; Zhao, Yu et al. 2007; Michael, Breton et al. 2008), thus prompting us to analyze if RGA protein levels and diurnal expression of GA-related genes is altered in *elf3-8* and *ELF3*ox seedlings. To this end, seedlings were grown under SD conditions, and samples were collected every 4 hours starting at ZT8, where ZT0 was defined as the preceding lights-on signal, before samples were started to be collected.

In western blot analyses with the anti-RGA specific antibody we observed that levels of the RGA repressor in *Col-0* seedlings increase just after dawn (ZT26), to subsequently decrease during the day and be maintained to low levels during night time (Figure 35a). Notably, constitutively elevated levels of this repressor were observed in *elf3-8* seedlings, while levels of this protein were reduced in *ELF3*ox plants, even they show still a similar diurnal pattern as seen in wild-type *Col-0* plants (Figure 35a). Hence, RGA protein levels in *elf3-8* and *ELF3*ox plants does not correlate with the tall and short hypocotyl phenotype of these seedlings (Figure 8), in apparent conflict with previously published work that directly associated RGA levels with hypocotyl growth (de Lucas, Daviere et al. 2008).

ELF3 is a central component of the EC complex, loss-of-function mutations in this gene leading to an altered pattern of expression of multiple evening repressed genes (Dixon,

Knox et al. 2011; Nusinow, Helfer et al. 2011; Chow, Helfer et al. 2012). Interestingly, GA-perception and biosynthetic genes are repressed at dusk (Michael, Breton et al. 2008) and thus it is possible that mis-regulated expression of these genes leads to an alteration in GA levels and to RGA stabilization in the *elf3-8* mutant.

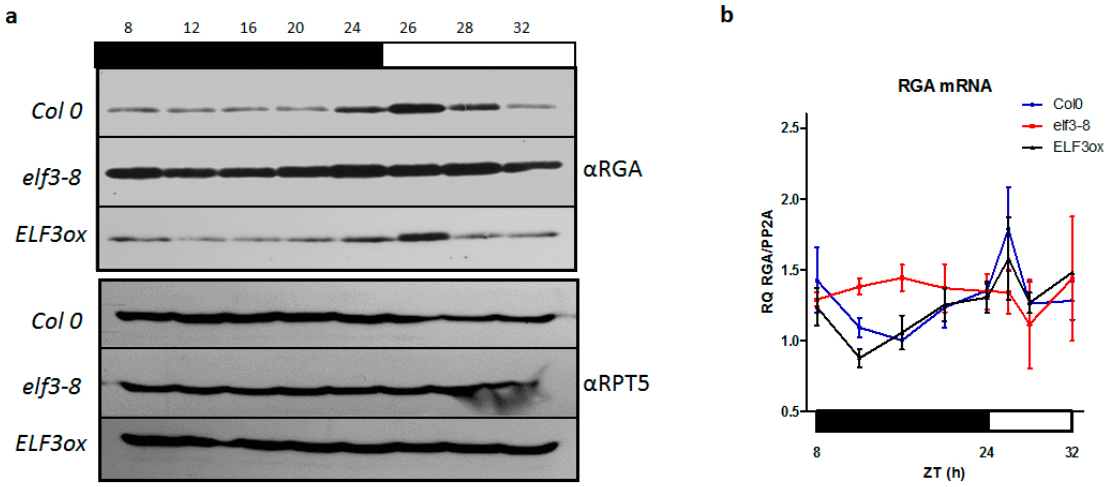

Figure 35. RGA levels are rhythmically modulated by ELF3. a) In seedlings grown in SDs, RGA is stabilized at dawn and levels of the protein are reduced during the day, the protein accumulating to low levels at night time. RGA protein levels are elevated in *elf3-8* seedlings and reduced in *ELF3*ox plants. b) Western blots were probed with the RPT5 antibody as loading control. c) Expression of the *RGA* gene shows a similar profile as the protein, with down-regulated levels of expression observed from evening to midnight followed by a peak of activation at dawn. *elf3-8* mutants show mis-regulated expression of this gene, with elevated levels of expression in the evening and early night, in agreement with a possible regulation of this gene by the EC. Data are the means and SD of three biological replicates.

Hence, we first set to analyze if *RGA* expression levels were changed in the *elf3-8* mutant and *ELF3ox* genetic backgrounds. As shown in Figure 35b, this gene was found to be repressed in *Col-0* seedlings in the evening, to reach lowest levels of expression by midnight and be again activated at dawn. This pattern of expression coincides with that reported in the DIURNAL database (Figure 36c,d) (Mockler, Michael et al. 2007; Michael, Breton et al. 2008), and reminds that of the RGA protein (Figure 35a). Interestingly, *ELF3*ox plants display a similar pattern of expression as *Col-0* but in *elf3-8* seedlings this gene is clearly misregulated as it shows increased levels of expression during evening and early night, and reduced activation at dawn (Figure 35b).

a

		Free running					Circadian					
	locus	DD _DDHC	LL23 _LDHH	LL12 _LDHH	LL _LDHC	LL _LLHC	LDHH _SM	LDHH _ST	longday	shortday	LDHC	LLHC
Circadian regulated	AT2G25930_ ELF3	nr	12	16	16	15	15	16	16	14	16	16
	AT2G43010_ PIF4	nr	5	7	7	9	7	6	7	4	6	9
Indirectly regulated	AT4G14130_ XTR7	nr	nr	nr	nr	11	nr	nr	nr	23	nr	9
	AT2G01570_ RGA	15	nr	nr	nr	nr	5	23	nr	23	nr	12

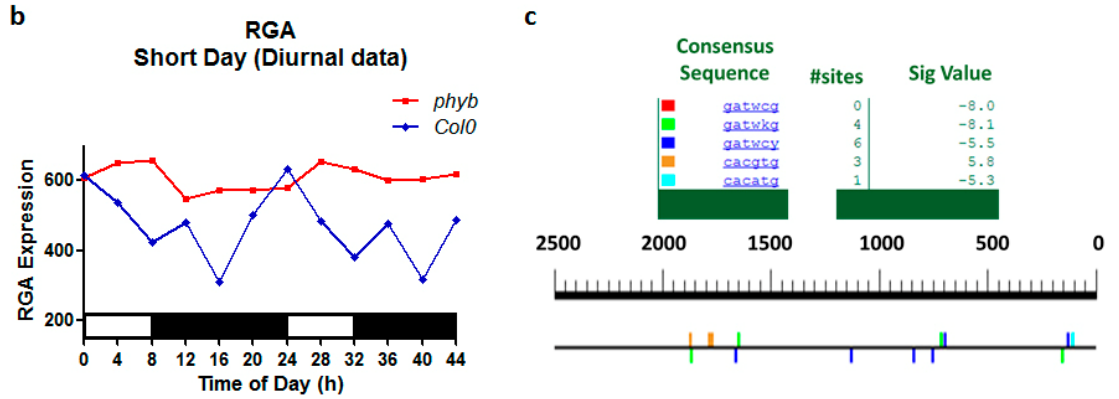

Figure 36. RGA expression is not directly regulated by the clock. a) Oscillation patterns of clock-regulated and non-clock regulated genes. While the clock-regulated *ELF3* and *PIF4* genes show a similar oscillation peak under diurnal an free-running conditions, the non-clock regulated *XTR7* and *RGA* genes fail to oscillate under free-running conditions. DIURNAL conditions are described in the Methods section. b) In the DIURNAL database *RGA* shows a similar expression profile in *Col-0* seedlings as that observed for the protein. Rhythmic expression of *RGA* is lost in *phyb* seedlings. c) The *RGA* promoter does not show any consensus LUX-binding site (red), although degenerated copies of this consensus motif are still present (green, blue). Several G-box (brown) and E-box (light blue) motifs bound by the PIF4 factor can be identified in the *RGA* promoter.

This diurnal pattern of expression, however, is not necessarily indicative of a regulation by the clock, as the *PIL1* and *XTR7* genes, for instance, show a rhythmic pattern of expression due to destabilization of the PIF4 factor in the light (Al-Sady, Ni et al. 2006; Nozue, Covington et al. 2007). Under continuous free running conditions or in the *phyB* mutant background these genes fail to show a rhythmic expression, indicating that their cyclic expression relies on a light component. Noteworthy, a similar behavior is also observed for *RGA* (Figure 36a) as this gene does not oscillate in *phyB* seedlings (Figure 36b). This indicates that RGA is not directly regulated by the clock but by a light regulated component and consistent with this notion, the promoter of this gene lacks any consensus LUX-binding site (Figure 36c). However, although *RGA* expression does not oscillate in the *phyb* mutant, increased levels of this transcript are observed in

this mutant background (Figure 36b), in a similar way as seen in *elf3-8* seedlings. Noteworthy, these two mutants display elevated levels of the PIF4 factor, due to impaired repression by the EC complex (Nusinow, Helfer et al. 2011) and impaired destabilization of the protein by the PHYB photoreceptor (Castillon, Shen et al. 2007). Thus, increased PIF4 levels may be responsible for increased *RGA* transcription in these plants. Indeed, recent ChIP-seq studies using PIF4-HA lines (Hornitschek, Kohnen et al. 2012; Oh, Zhu et al. 2012) showed that the PIF4 factor binds to several promoters of the DELLAs and GA biosynthetic genes (Table 10) (Hornitschek, Kohnen et al. 2012). These data in combination with gene expression studies of lines expressing the PIF4 factor under control of its native promoter (Oh, Zhu et al. 2012) showed that the *RGA, GAI, GA3ox1,* and *RGL1* genes are direct targets for PIF4-regulation (PRPT), that the *GID1a, RGL3, GA2ox1,* and *GA20ox1* genes are PIF4 regulated, but are not direct targets of this regulator, whereas *RGL2* and *ELF3* are not regulated by PIF4 (Table 10). Likewise, ChIP-seq studies of *35S::*PIF5-HA lines, in combination with microarray data conducted after two hours of treatment with low R/FR light, identified the *RGA*, *GAI*, *GID1a* and *GA2ox6* genes as direct targets of the PIF5 factor and most likely also of PIF4. Moreover, PIF5 was found to directly regulate the *PIF4* gene and an *ELF3* homolog (At3g21320) lacking the N-terminal conserved domain.

In the gene expression analyses shown in Figure 35b, levels of the *RGA* transcript were not much different between *Col-0* and *elf3-8* seedlings, being even inverted by the end of the day, which corresponds to the day interval in which PIF4 is active, as deduced from the peaks of *PIL1* and *XTR7* transcripts. This argues against a direct role of PIF4 in mediating increased levels of accumulation of the RGA repressor in the *elf3-8*, consistent with this notion, *RGA* expression levels were not as strongly reduced in *pif4pif5* mutant seedlings (Figure 37a) as seen for *PIL1* gene expression (Figure 10), although we cannot exclude that other PIFs contribute to maintain elevated levels of expression of this gene. *RGA* expression, on the other hand, was found to be increased in *PIF4ox* lines, consistent with the observation that several PIF4 recognition motifs are present in the promoter of this gene (Hornitschek, Kohnen et al. 2012).

Table 10. PIF-regulated PIF4 target genes relevant to this work. The GA signaling genes *RGA*, *GAI*, *RGL1*, *GID1a* and *GA3ox1* were identified as direct PIF4 targets in PIF4 ChIP-seq analyses, but only *RGA* and *GAI* could be identified in a second independent study studies (Hornitschek, Kohnen et al. 2012) and (Oh, Zhu et al. 2012). CBF3, CBF1 and FT were reported to be PIF4 regulated in response to increased temperature (Lee and Thomashow 2012), but were not identified in microarray or ChIP-seq studies. PIF4 binds the clock genes *CCA1*, *LHY*, *AtPRR9*, and *TOC1* promoters, but expression of these genes does not change in *PIF4ox/pif4*

	Gene	Total	Gene	Total	Gene
GA signaling	**RGA**	Up	RGL2	up	RGL3
	GAI	Up	GA20ox1	up	
	RGL1	Up	**GID1a**	up	
	GA3ox1	Up	GA2ox1	up	
PIF4 known targets	ATHB2	Up			CBF3
	XTR7	Up			CBF1
	IAA19	Up			FT
	PIL1	Up			
	CBF2	Up			
Clock genes	CCA1	Complex	TOC1	complex	ELF3
	LHY	Down	CAB1	down	GI
	APRR9	Complex			CO

Since PIF4 protein stability is increased under FR light conditions, we decided to analyze levels of the RGA protein in *elf3-8*, *elf3-8pif4pif5, pifq* and *PIF4ox* seedlings grown in cR light, after a shift to FR light. Remarkably, *pifq* seedlings showed reduced RGA levels compared to the wild-type *Col-0* background, while *PIF4*ox lines showed increased levels of this repressor with respect to the wild-type controls. However, transfer to FR conditions induced an accumulation of the RGA protein in all three genetic backgrounds, hence indicating that although the PIF4 factor contributes to *RGA* expression is not the main factor mediating accumulation of the RGA protein. In this sense, it is important to note that RGA protein levels were strongly increased in *elf3-8*

and *elf3-8pif4pif5* seedlings and that levels of this protein were not further increased by FR light conditions (Figure 37b), hence suggesting that ELF3 plays a more prevalent role in regulating RGA protein levels than the PIF4 factor. Intriguingly, *elf3-8* seedlings show elongated hypocotyls, while the hypocotyls of *elf3-8pif4pif5* seedlings are small, and both plants accumulate high levels of the RGA repressor. This would suggest that this repressor is inactive in the *elf3-8* mutant or alternatively, that RGA is unable to restrict hypocotyl growth in the absence of ELF3. Other possibility is that ELF3 is exerting its function over RGA in other tissue than the hypocotyl.

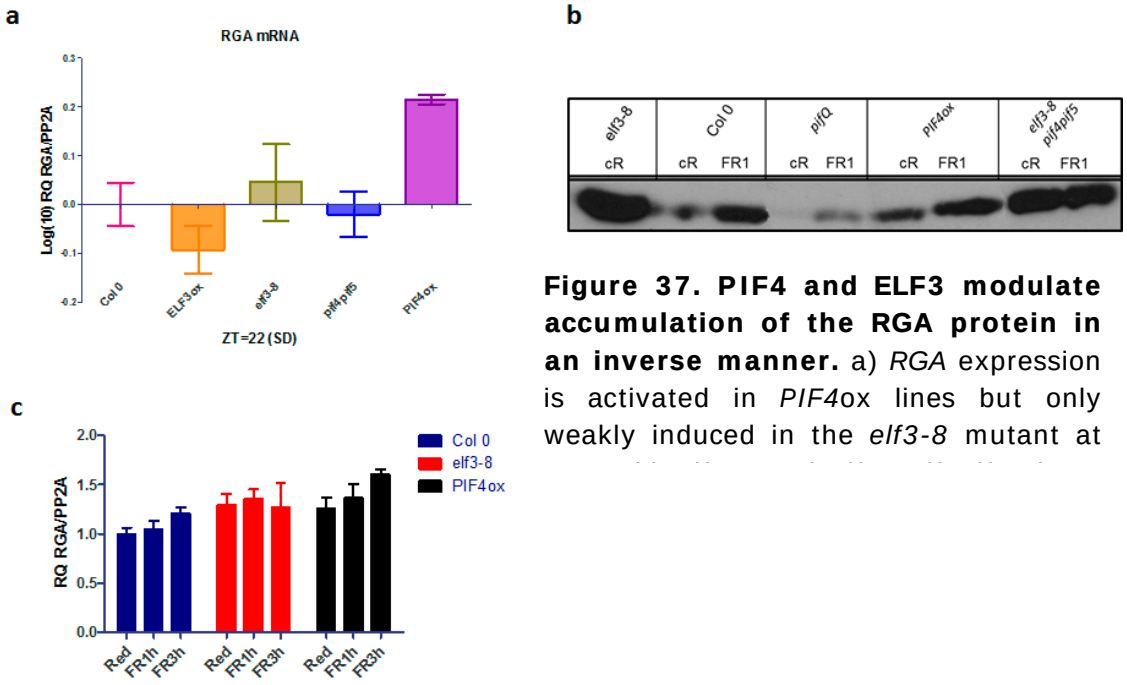

Figure 37. PIF4 and ELF3 modulate accumulation of the RGA protein in an inverse manner. a) *RGA* expression is activated in *PIF4*ox lines but only weakly induced in the *elf3-8* mutant at

Increased RGA protein levels in FR light, in the *Col-0*, *pifq* and *PIF4*ox backgrounds, on the other hand, do not correlate with increased levels of transcription of this gene (Figure 37c), suggesting that accumulation of this repressor is mediated by a post-transcriptional regulatory mechanism. Also, the finding that *elf3-8* seedlings show a misregulated pattern of expression of the *RGA* gene, with elevated levels of this transcript in the night, but reduced transcript levels during the day, suggests that feed-back regulation of GA signaling is altered in these plants. Therefore, we set to analyze levels of the RGA protein in plants in which transcription had been blocked by cycloheximide and to further test if expression of the GA biosynthetic and catabolic genes is altered in the *elf3-8* mutant.

ELF3 modulates RGA stability independently of transcription.

In *Col-0* seedlings, RGA protein levels peak directly after lights-on, to be subsequently reduced during the day and reach basal levels during night time (Figure 35a). To assess if this oscillation is associated to changes in the transcription rate or to a differential stability of the protein, we treated the seedlings with 50μM cycloheximide (CHX) at ZT4 (Figure 38a), at this time point RGA starts to decrease) to further analyze RGA protein levels at different time points after CHX application. As seen in Figure 38b, treatment with CHX completely blocked RGA accumulation in *Col-0* and *ELF3*ox seedlings but remarkably, high levels of the protein were still observed in the *elf3-8* mutant background, hence demonstrating that this repressor is strongly stabilized in the absence of ELF3. To further assess if GA-induced destabilization of DELLAs is impaired in the *elf3-8* plants, seedlings were treated with CHX in the presence of 25 μM GA_3. As shown in Figure 38c, GA application led to a rapid destabilization of the RGA protein in all genetic backgrounds, hence excluding an impaired GA response in the *elf3-8* mutant, or the lack of expression of the F-box SLY1 protein in these plants.

Together, these findings demonstrate that RGA stability is strongly increased in *elf3-8* plants, stabilization of this protein being independent of *de-novo* protein synthesis. However, the fact that *elf3-8* mutants show a tall phenotype, despite accumulating high levels of the RGA repressor, raises the question if this protein is biologically active. At least for RGL2, substitution of a conserved Thr by an Asp residue ($RGL2^{T272D}$), mimicking a constitutive phosphorylation, leads to a strong stabilization of the protein, but this protein was shown to be unable to repress *GA20ox* expression in tobacco BY2 cells (Hussain, Cao et al. 2005).

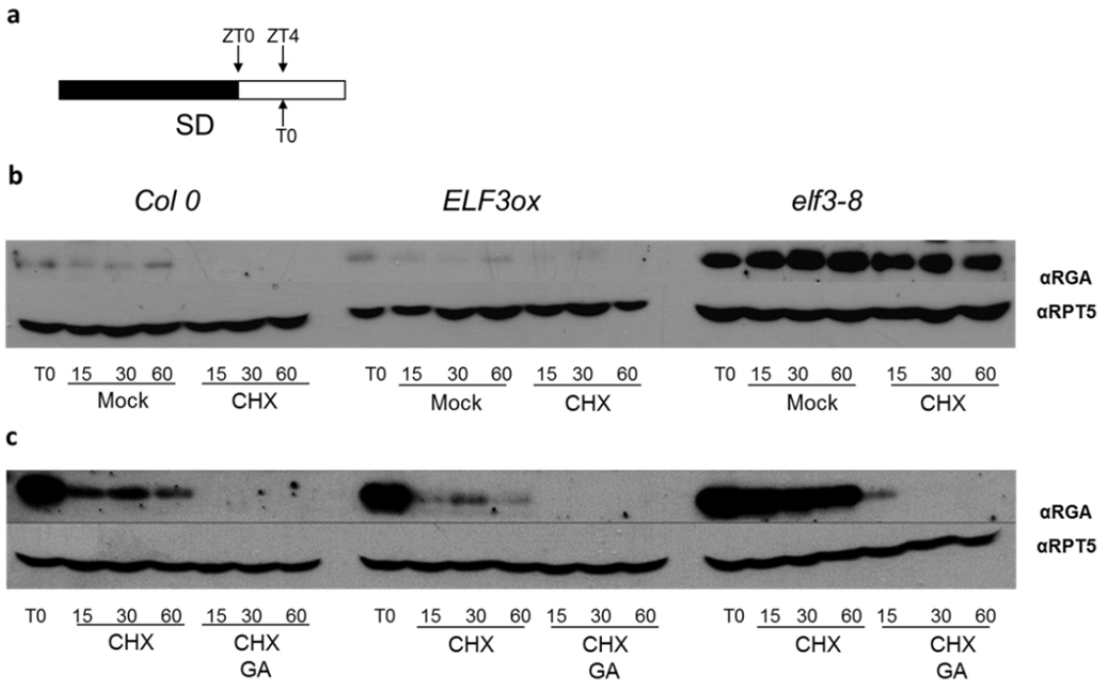

Figure 38. ELF3 modulates RGA protein stability independently of gene expression. a) Seedlings were grown in SDs and treated with CHX at ZT4, which is the time point where RGA protein levels start to decrease. b) CHX application blocks RGA accumulation in *Col-0* and *ELF3*ox plants. However, high levels of the protein are still observed in *elf3-8* seedlings. c) GA-induced degradation of DELLAs is not impaired in the *elf3-8* mutant. Seedlings were treated with 50µM CHX and 25µM GA_3.

elf3-8 loss-of-function impairs feed-back regulation of GA synthesis.

Endogenous GA levels are regulated by a feedback control mechanism involving a negative regulatory loop of the GA biosynthetic genes and feed-forward regulation of the DELLAs and GA catabolic enzymes. Genes encoding the last biosynthetic steps (*GA20ox, GA3ox*) and the GA receptors (*GID1a/b*) are down-regulated in the *della* mutant or upon GA application, while application of GAs induces expression of the DELLA genes and the *GA2ox* catabolic enzyme (Xu, Li et al. 1995; Cowling, Kamiya et al. 1998; Thomas, Phillips et al. 1999; Xu, Li et al. 1999; Dill and Sun 2001; Zentella, Zhang et al. 2007). Levels of expression of the metabolic enzymes (*GA20ox, GA3ox*) and *GID1* genes are, on the other hand, elevated in the GA deficient *ga1-3* mutant and in *rgaΔ17* seedlings, expressing a stable form of the RGA repressor, while those of the catabolic enzymes (*GA2ox*) and DELLA genes are sensibly reduced (Xu, Li et al. 1995; Cowling, Kamiya et al. 1998; Thomas, Phillips et al. 1999; Xu, Li et al. 1999; Dill, Jung et al. 2001; Dill and Sun 2001; Zentella, Zhang et al. 2007). During

photomorphogenesis, phytochrome modulation of bioactive GA levels is also thought to restrain hypocotyl growth via stabilization of the DELLAs (Achard, Liao et al. 2007). In a shift from dark to light, expression of *GA2ox1* increases and RGA accumulates but *GID1a/b* and *RGA* expression is not affected. *GA20ox* and *GA3ox* gene expression is reduced in the first two hours of light, to start later increasing, whereas during light to dark transition, *GA2ox1* expression is repressed and RGA destabilized. This shift does not modify *RGA* and *GID1a/b* gene expression, but expression of the *GA20ox1* and *GA3ox1* genes is slightly reduced (Achard, Liao et al. 2007).

Since DELLAs accumulate to high levels in the *elf3-8* mutant, we decided to analyze levels of expression of the *GID1* receptor and the biosynthetic and catabolic enzymes, to assess if GA homeostasis is perturbed in these plants. Thus, qPCR studies were conducted in *Col-0*, *ELF3*ox and *elf3-8* plants, to analyze diurnal levels of expression of these genes.

As seen in Figure 39a, the *GA20ox1* biosynthetic gene shows in *ELF3ox* and *Col-0* seedlings a diurnal pattern of expression characterized by low levels of transcript in the night and activated expression in the day, to peak around midday with similar levels in all three backgrounds. Interestingly, this gene was found to be strongly up-regulated in the night in the *elf3-8* mutant background, but to be repressed with the lights-on signal, to follow during the day a similar pattern as seen in the *ELF3*ox and *Col-0* plants. In *Col-0* seedlings, the *GA3ox1* gene shows a similar pattern of expression, with reduced transcript levels at night and a peak of expression at midday (ZT4) (Figure 39b). In *elf3-8* seedlings, this gene is up-regulated during early night but repressed during the second half of the night, to peak again during the day as seen in *Col-0* plants. In opposite to wild-type, however, transcript levels did not decrease by the end of the day in the *elf3-8* mutant, with relatively high transcript levels observed at dusk and early night. Intriguingly, expression of this gene was found also to be up-regulated in *ELF3ox* lines, with a peak of expression at late night, and a higher peak of expression during the day. Expression of the *GA2ox1* catabolic enzyme, in turn, was reduced at night and peaked with the lights-on signal, to be again down-regulated during the day (Figure 39c). This gene showed a similar expression profile in all three genetic backgrounds, although it peaked to a much higher levels in the *elf3-8* mutant. Notably, peak of expression of this gene coincides with the peak of accumulation of the RGA protein (Figure 35a), indicating that bioactive GA levels are most likely reduced at this point of the day. Concerning *GID1a* expression, transcripts for this gene peaked during early night to be steadily reduced and reach lowest levels during early day, and be subsequently activated towards the night (Figure 39d). This rhythmic expression is lost

in *elf3-8* mutants, with these plants showing elevated transcript levels all over time points. In *ELF3*ox lines, *GID1a* expression is reduced during the first half of the night, but shows a similar pattern as the wild-type during the rest of the day. Noteworthy, lowest levels of *GID1a* expression coincide with the peak of accumulation of the RGA protein, in agreement with previous reports showing that diurnal expression of GID1 plays a prevalent role in DELLAs stabilization and in rhythmic growth (Arana, Marin-de la Rosa et al. 2011; Marin-de la Rosa, Alabadi et al. 2011). *GA20ox1*, *GA3ox1* and *GA2ox1* expression is also high during the day, when *GID1a* expression is lowest. However, these genes peak at different times of the day, the *GA2ox1* peak being observed immediately after dawn, whereas *GA20ox1* and *GA3ox1* peak at midday. In *elf3-8*, RGA accumulates at more or less constant levels, with the *GA20ox1, GA3ox1* genes being expressed to higher levels in the night and expression of the *GID1a* gene being no longer rhythmic, which is indicative of altered GA homeostasis. Noteworthy, *GA2ox1* expression is also up-regulated in these plants (Figure 39d), which suggests that bioactive GA levels might be reduced in these plants, thus contributing to RGA stabilization. However, this is in apparent contradiction with the up-regulated levels of expression of the *GA20ox1* and *GA3ox1* genes, which would lead to increased bioactive GA synthesis. Hence, altered GA homeostasis is likely to result from impaired destabilization of the DELLAs, which mimics “low GA” conditions. Enhanced RGA destabilization in *ELF3*ox lines explains also in part the repression of *GID1a* expression during early night, but not the rather erratic pattern of *GA3ox1* expression seen in these plants.

Interestingly, RGA protein levels peak at the point of lowest *GID1a* expression and this coincides with a decline in the levels of expression of the PIF4 target *XTR7* and *PIL1* genes (Figure 9c,d). This indicates that RGA likely represses the activity of PIF4 during this interval of the day, until enough Pfr phytochrome is translocated to the nucleus to destabilize this factor. The diurnal pattern of the *GID1a*, *GA20ox1*, *GA3ox1* and *GA2ox1* genes, on the other hand, seems to result more from diurnal oscillations in GA levels and RGA protein stability, than from regulation by the clock or by PIF4-dependent expression. Actually, feed-back studies of these genes were mostly performed in continuous light, and it is possible that under photoperiodic conditions PIF4 and RGA-dependent regulation of these genes overlap, leading to a complex pattern of expression.

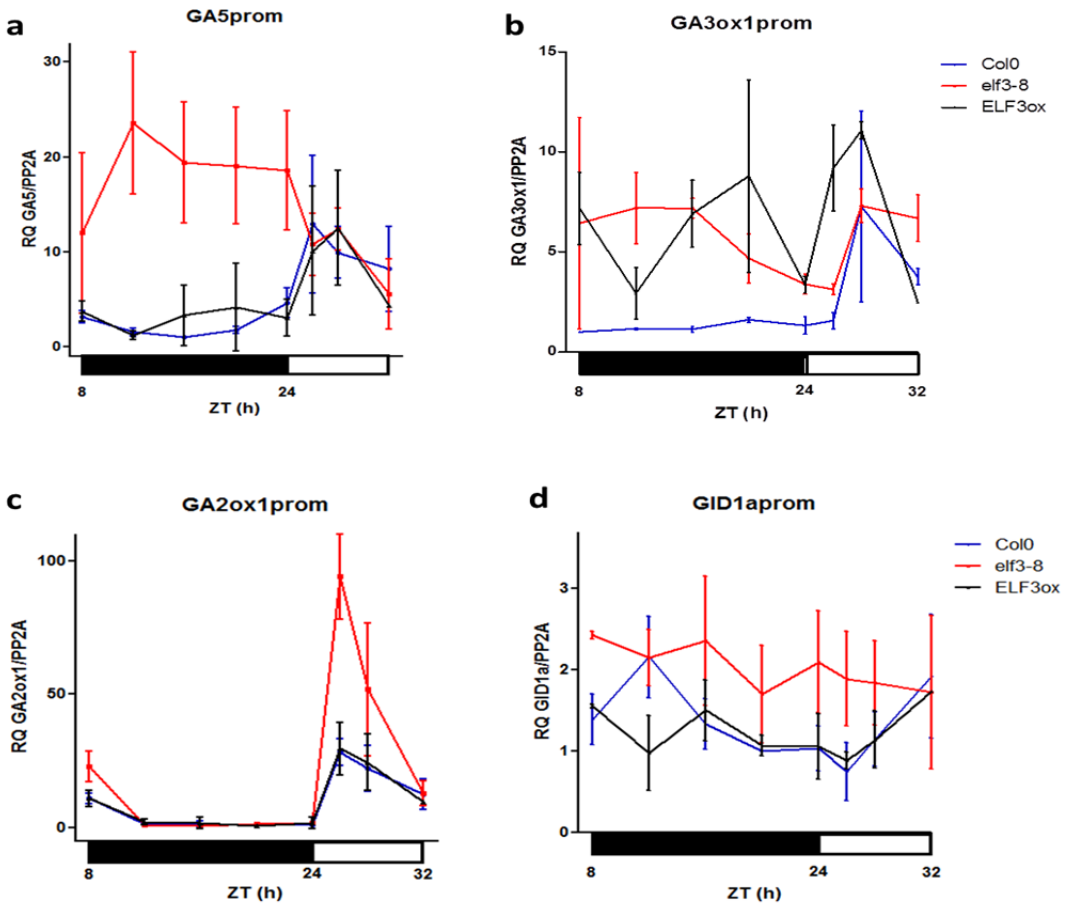

Figure 39. GA biosynthetic genes are misregulated in *elf3-8* plants. The GA biosynthetic a) *GA20ox1* and b) *GA3ox1* genes are misregulated in elf3-8 seedlings in the night. In *ELF3*ox plants the *GA3ox1* gene is also up-regulated but shows an erratic pattern of expression. c) The *GA2ox1* catabolic gene peaks at dawn, coinciding with the peak of accumulation of the RGA protein. This gene is strongly induced in the *elf3-8* mutant. d) *GID1a* losses its rhythmic expression in *elf3-8* seedlings. In ELF3ox lines this gene is repressed during early night, but expression is maximal at this point in *Col-0* wild-type plants. The results are the average of three biological replicates.

Over-expression of *GID1a*, on the other hand, was reported to repress RGA activity independently of endogenous GA levels (Ariizumi, Murase et al. 2008). Loss-of-function alleles of the F-box *SLY1* gene actually display a milder dwarf phenotype than the *ga1-3* mutants, although similar levels of stabilization of the RGA repressor are observed in both plants. GID1a over-expression was found to rescue the dwarf phenotype of *sly1-10* seedlings, without a concomitant decrease in DELLA levels, indicating that formation of the RGA-GID1a complex inactivates the RGA repressor via a destabilization-independent mechanism (Ariizumi, Murase et al. 2008; Ueguchi-Tanaka, Hirano et al. 2008). Thus, we cannot rule out that elevated *GID1a* gene

expression in the *elf3-8* mutants, contributes to inactivate the RGA protein.

ELF3 regulates RGA levels in the cotyledons.

A still intriguing question was whether the RGA repressor is active in the *elf3-8* mutant, since the tall hypocotyl phenotype of these plants is in contradiction with the characteristic dwarf phenotype of DELLA accumulating mutants. Expression of the *GID1a* and GA biosynthetic genes was found to be altered in these seedlings, hence suggesting that RGA is indeed biologically active. However, samples collected for both protein and qPCR studies were whole seedling and thus it is still possible that RGA accumulates in a differential manner in these plants, such that the protein accumulates to lower levels in the hypocotyls compared to the cotyledons. DELLAs have actually been shown to be implicated in cotyledon growth inhibition (Penfield, Gilday et al. 2006; Josse, Gan et al. 2011), and it is unclear if ELF3 shows a differential distribution in the plant. In consequence, we dissected cotyledons from the hypocotyls in *Col-0*, *ELF3*ox and *elf3-8* seedlings, to analyze RGA repressor levels in these organs. As shown in Figure 40a, b, western blot studies of these organs showed that in RGA accumulates in the *elf3-8* cotyledons, but not in the hypocotyls. Likewise, in *Col-0* seedlings ELF3 is enriched in the cotyledons but is not detected in the hypocotyls (only an unspecific band of slightly higher molecular weight is detected in the hypocotyls. This band is also observed in *elf3-8* hypocotyls, which demonstrates that it does not correspond to the ELF3 protein. Notably, differential distribution of the ELF3 and RGA proteins correlates with the smaller cotyledons of *elf3-8* seedlings and the larger cotyledon size of *ELF3*ox plants (Figure 40c, e). Actually, while *elf3-8* plants have taller hypocotyls (Figure 40d), their cotyledons are smaller (Figure 40c, e), whereas *ELF3*ox plants display shorter hypocotyls but larger cotyledons, hence demonstrating that ELF3 plays a prevalent role in modulating RGA levels only in the cotyledons.

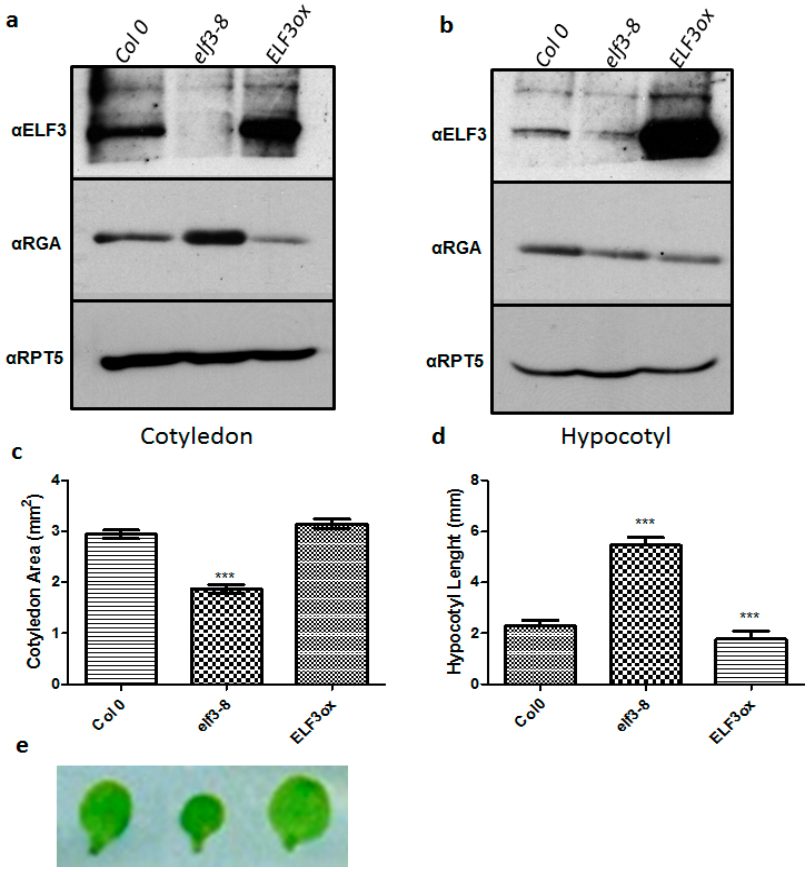

Figure 40. RGA accumulates in the cotyledons of *elf3-8* seedlings. Seedlings where dissected to separate the cotyledons from the hypocotyls, while roots were discarded. Protein extracts of these organs were probed with anti-ELF3 and anti-RGA antibodies to analyze the levels of these proteins. a) RGA levels are increased in the cotyledons but not in the b) hypocotyl of *elf3-8* plants. Likewise, the ELF3 protein is enriched in *Col-0* cotyledons but is not detected in the hypocotyls. c) *elf3-8* seedlings show smaller cotyledons than the wild-type, while cotyledons of *ELF3*ox plants are larger. d) Hypocotyls are taller in *elf3-8* seedlings and shorter in *ELF3*ox lines. e) Representative picture of the cotyledon size of these plants.

Co-expression of COP1 affects the nuclear pattern of localization of the RGA and ELF3 proteins.

ELF3 has been shown to interact with the E3 ligase COP1 via the N-ELF3 domain, to direct proteasome degradation of the GIGANTEA (GI) protein that binds the M-ELF3 domain (Yu, Rubio et al. 2008). Since RGA binds also the M-ELF3 domain, ELF3 might play a similar role in destabilizing this repressor protein, by serving as a scaffold protein for COP1 and RGA interaction. Actually, ELF3 co-localizes with COP1 in nuclear speckles (Yu, Rubio et al. 2008) and as shown in Figure 31b, we had observed that ELF3 and RGA interact in the nucleus. Therefore, we decided to test if co-expression

of COP1 drives localization of the RGA repressor into nuclear speckles. To this aim, MYC, YFP and CFP fusion constructs of the COP1, ELF3 and RGA proteins were expressed in *N. benthamiana* leaves to test if co-expression of these proteins modified their nuclear localization pattern. As shown in Figure 41a, YFP fluorescence of the COP1-YFP protein was, as reported (Wang, Ma et al. 2001), distributed in nuclear speckles (Figure 41a). Fluorescence of the ELF3-YFP protein, by contrary, was found to be homogeneously distributed in the nucleus (Figure 41b), in opposite to previous reports showing a nuclear speckle localization of the protein (Herrero, Kolmos et al. 2012). Remarkably, we used the *Col-0* allele to generate the ELF3-YFP fusion, while the *Ws* allele was used in the reported studies. These two alleles differ in the length of a poly-Q stretch (7 Q in *Col-0*, and 16 Q in *Ws*) (Coluccio, Sanchez et al. 2011) (Figure 41f), with differences in this poly-Q stretch being also reported for the *Bay* and *Sha* alleles, these allelic differences being thought to underlie differential response of these ecotypes to shade (Jimenez-Gomez, Wallace et al. 2010; Coluccio, Sanchez et al. 2011). Notably, a similar polymorphism in a poly-Q stretch is also observed in the two potato copies of the CONSTANS gene, with variations in this region determining a differential distribution of these proteins in a diffuse or nuclear speckle pattern (Abelenda J.A, personal communication). Therefore, it will be interesting to conduct future studies to analyze if polymorphisms in this region lead to differential activity/nuclear localization of these ELF3 allelic forms. Apart from this discrepancy, we observed that when ELF3-YFP is co-expressed with the COP1-MYC protein, YFP fluorescence is no longer diffuse but forms nuclear speckles (Figure 41c). Also, when ELF3-YFP is co-expressed with the RGA-CFP fusion, YFP fluorescence is diffuse (Figure 41d), but ELF3-YFP localization changes into nuclear bodies of larger size, upon additional expression of the COP1-MYC protein (Figure 41e).

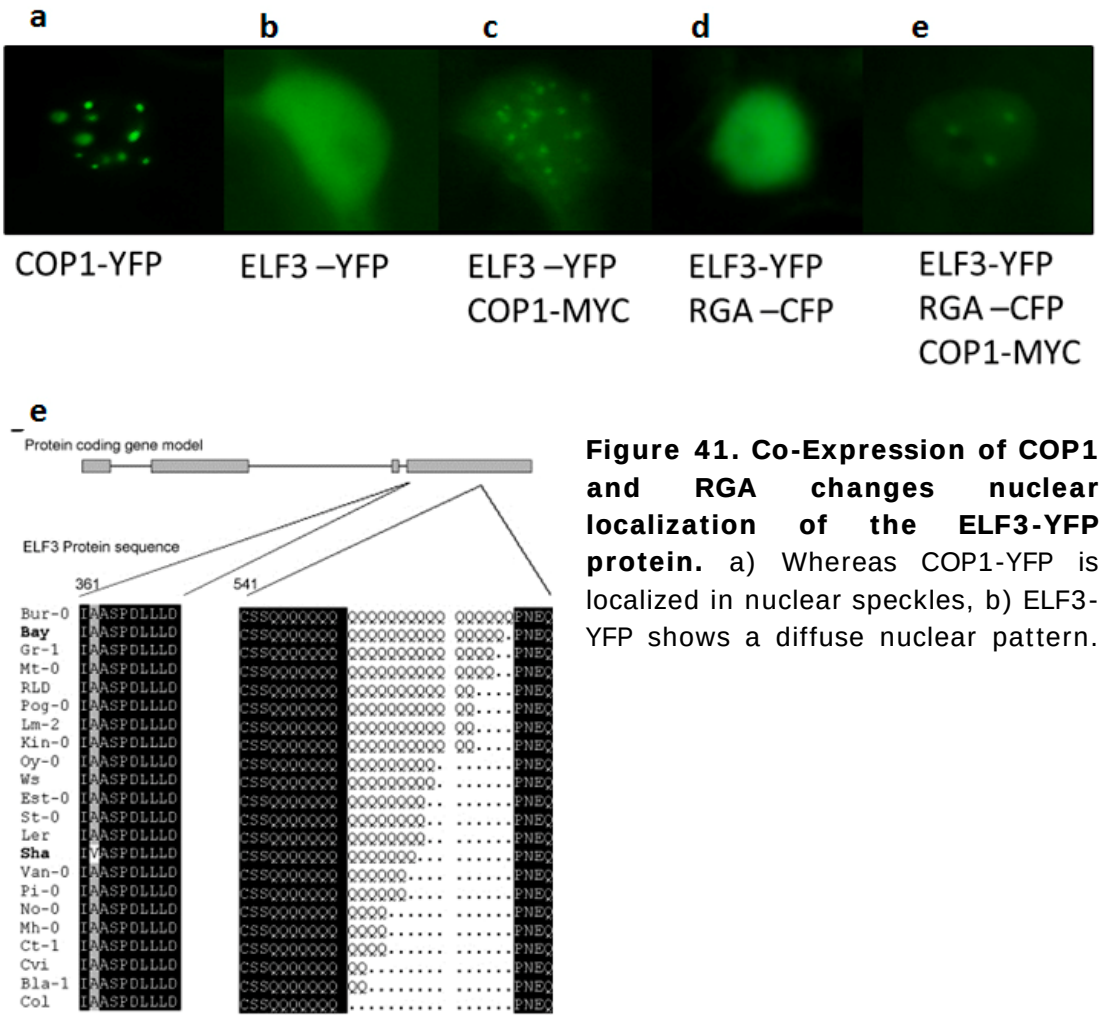

Figure 41. Co-Expression of COP1 and RGA changes nuclear localization of the ELF3-YFP protein. a) Whereas COP1-YFP is localized in nuclear speckles, b) ELF3-YFP shows a diffuse nuclear pattern.

RGA, on the other hand, was found to be homogeneously distributed in the nucleus (Figure 42a, (Silverstone, Ciampaglio et al. 1998)). As co-expression of this protein, along the COP1-MYC protein, modifies the nuclear localization pattern of the ELF3-YFP fusion, we wanted to test if this correlates also with a change in the nuclear distribution of the RGA protein. To this aim, we co-expressed a RGA-YFP construct with the ELF3-HA and COP1-MYC proteins, as we were unable to detect the fluorescence of the cyan RGA-CFP construct. As shown in Figure 42b and c, the nuclear distribution of the RGA-YFP protein did not change upon expression of either the ELF3-HA or COP1-MYC proteins alone, but RGA-YFP localized in nuclear bodies when co-expressed with the ELF3 and COP1 proteins (Figure 42d), which suggests that RGA, ELF3 and COP1 co-localize in these nuclear bodies.

Levels of the RGA protein are actually increased in *cop1-4* seedlings (Figure 32), which is suggestive of a role of COP1 in DELLA destabilization. In western blot studies of the

infiltrated leaves, reduced levels of the RGA-YFP protein are in fact observed after co-expression with the COP1 and ELF3 constructs, but not when expressed with the COP1 protein alone (Figure 42j), indicating that ELF3 is required for COP1-mediated destabilization of this repressor.

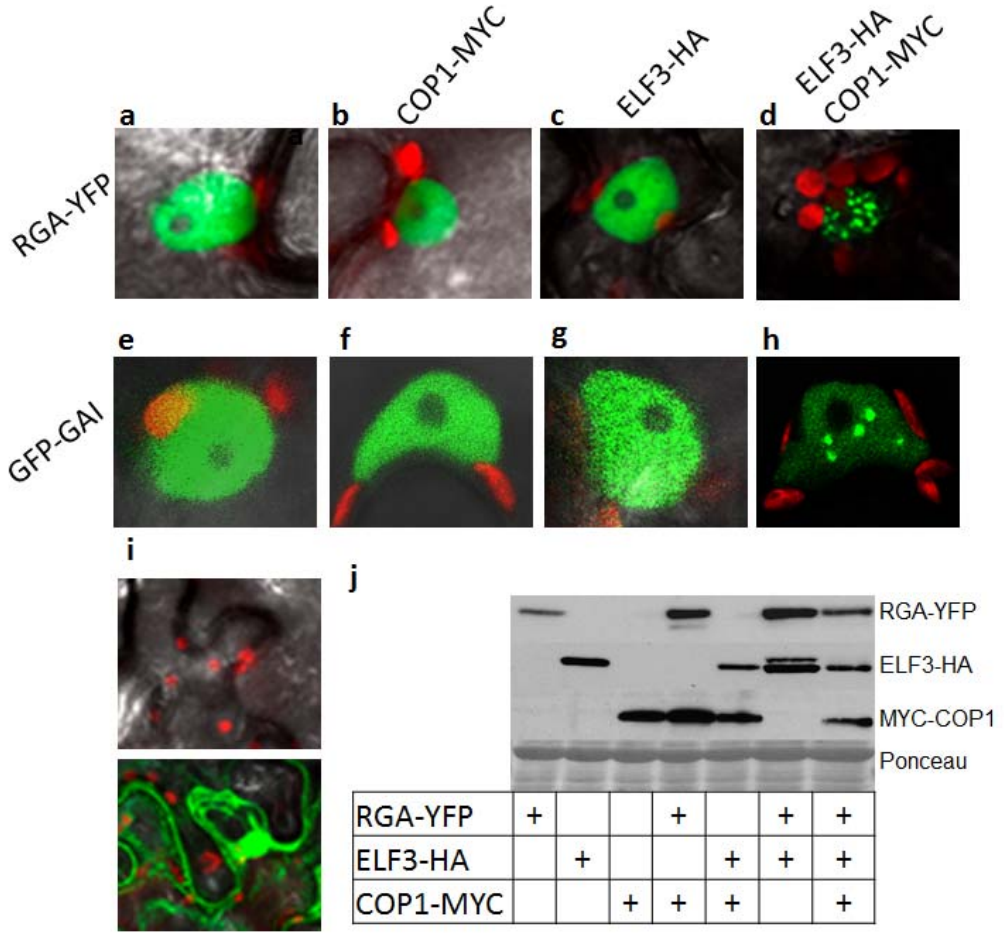

Figure 42. Co-Expression of ELF3 and COP1 changes the nuclear localization pattern of the RGA and GAI proteins. a) RGA and e) GAI are nuclear localized and show a diffuse pattern of distribution. Neither COP1, b, f), nor ELF3 alone, c, g), change the nuclear pattern of distribution of these DELLA proteins. However, in the presence of ELF3 and COP1, the d) RGA and h) GAI proteins localize into nuclear bodies. i) No GFP signal is detected in the negative controls without GFP. Co-expression of the COP1 and ELF3 proteins does not change ubiquitous distribution of a MYC-GFP fusion protein. j) COP1 leads to a reduction in ELF3-MYC protein levels and a reduction in the levels of accumulation of the RGA-YFP protein is also observed upon co-expression of both COP1 and ELF3

Noteworthy, co-expression of COP1 was reported to lead to a strong reduction in ELF3 protein levels when these proteins are co-infiltrated in *benthamiana* leaves (Liu, Zhang

et al. 2010) and a reduction in ELF3-HA levels is also observed in leaves co-expressing the COP1-MYC and ELF3-HA proteins (Figure 42j). However, such reduction is not as strong as previously reported, which indicates that the conditions we used for these studies were not the optimal to see a destabilization of these proteins. Regardless that, a similar change in GFP-GAI distribution was observed after co-expressing this DELLA with the ELF3 and COP1 proteins (Figure 42e-h), hence pointing to a prevalent role of the ELF3 protein in the formation of a ternary DELLA-ELF3-COP1 complex implicated in DELLAs destabilization. Together, these results indicate that ELF3 serves as a scaffold protein to direct COP1-mediated destabilization of the RGA protein and to confirm such a regulatory model we crossed *pRGA*::GFP-RGA *Col-0* lines with *elf3-8*, *COP1*ox, *cop1-4, PHYB*ox and *phyB* seedlings, to analyze RGA protein stability in these genetic backgrounds.

ELF3 and COP1 modulate stability of the RGA repressor in an organ-specific manner.

Cryptochromes were reported to regulate expression of the GA biosynthetic genes under prolonged blue light but surprisingly GA levels of *cry1cry2* mutants were similar to those of wild-type plants (Zhao, Yu et al. 2007). As pointed by the authors, lack of changes in endogenous GA levels indicates that GA biosynthetic genes are activated in a tissue-specific manner in these mutant plants. Cryptochromes were actually reported to inhibit COP1 E3 ligase activity, by directly binding the COP1 protein, which competes for SPA1 interaction, to block COP1-SPA1 ubiquitination activity (Fankhauser and Ulm 2011). The finding that RGA accumulates to higher levels in *cop1-4* seedlings actually suggests that this repressor is one of the ubiquitination targets for COP1 activity, with ELF3 and COP1 exerting similar negative effects on RGA stability during the switch from light to darkness, but exerting an independent regulatory control in darkness, and during the switch from dark to light. Hence, to obtain insights on a possible regulatory role of COP1 over the RGA protein, independent of ELF3 function, we assessed if these protein are to directly interact in yeast cells. Due to technical difficulties we were unable to express the COP1 protein in a yeast system and therefore we used the *N. benthamiana* BiFC system to test interaction of these proteins. As shown in Figure 43, by expressing the RGA-nYFC and COP1-cYFC fusions in *N. benthamiana* leaves we were able to observe that these proteins interact in tobacco cells, and localize in nuclear speckles. This localization was different from that observed in Figure 42, where fluorescence of the RGA-YFP protein co-expressed with COP1-MYC showed a diffuse pattern and a nuclear body

distribution was only observed after additional expression of the ELF3-HA protein. However, with this system we cannot exclude that the endogenous tobacco ELF3 protein still acts as an adaptor bridge for COP1 and RGA interaction.

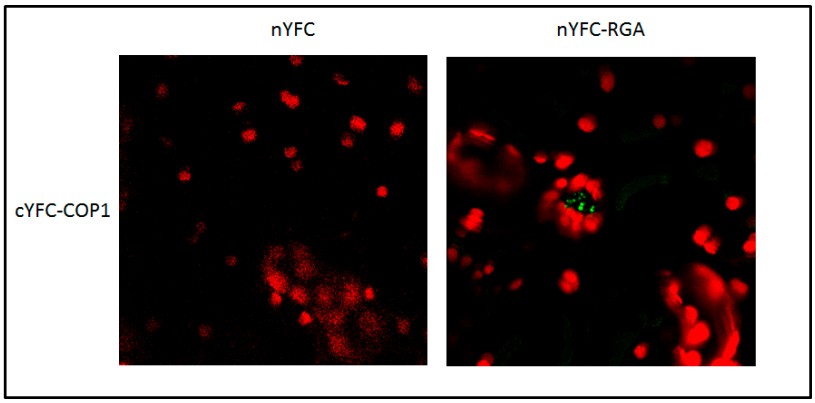

Figure 43. COP1 interacts with RGA in plant cells. Nuclear speckle YFP fluorescence is observed in leaves expressing the nYFC-RGA and cYFC-COP1 constructs, but not in control leaves infiltrated with either of these constructs and the complementary BiFC empty vector. The respective control cYFC with nYFC-RGA yielded similar results as in figure 31.

Regulation of RGA stability has been extensively studied in transgenic *pRGA::*GFP-RGA lines, expressing a GFP fusion of this repressor protein, under the control of its own promoter. These transgenic lines have been strategic to establish GA-dependent degradation of the DELLAs (Dill, Jung et al. 2001) and to show auxin-dependent destabilization (Fu and Harberd 2003), and ABA and ethylene modulation of these repressors (Achard, Vriezen et al. 2003; Achard, Cheng et al. 2006), among others. Transgenic *Col-0* lines bearing this construct were obtained from Dr. Tai-ping Sun, and crossed with *elf3-8*, *cop1-6*, *COP1*ox, *phyB* and *PHYB*ox lines. Hence, homozygous lines were generated which carried the same *pRGA*::GFP-RGA insertion in these different backgrounds, thus providing a very powerful tool for the analysis of DELLA regulation in response to PHYB, COP1 or ELF3 activities, or to immunoprecipitation studies to determine tissue-specific function of these proteins.

Preliminary confocal microscopy analyses of the levels of accumulation of the GFP-RGA protein in seedlings grown in continuous red light, confirmed that levels of this repressor are increased in *cop1-6* and *elf3-8* seedlings and reduced in *COP1*ox lines, as seen in western blot studies of whole seedling protein extracts (Figure 44 a-h). However, GFP fluorescence of this protein was not evenly distributed in *elf3-8* seedlings, showing strong GFP activity in the cotyledons but not in the hypocotyl

(Figure 44b, f), while in *cop1-6* seedlings, a strong GFP activity was detected in the hypocotyl in less extent in the cotyledons (Figure 44d, h). Noteworthy, this pattern of RGA accumulation nicely correlates with the phenotypes of these mutants (Figure 44i-j), as *elf3-8* seedlings show a tall hypocotyl but a reduced cotyledon size, whereas *cop1-6* seedlings show small hypocotyls but slightly larger cotyledons (Hsieh, Okamoto et al. 2000). *COP1*ox lines, on the other hand, show reduced GFP-RGA levels in all organs and display taller hypocotyls and a larger cotyledon size.

When kept in darkness, hypocotyl and cotyledon tissues undergo opposing growth responses, suggesting that DELLA proteins may be subject to differential regulation in these distinct tissues (Josse, Gan et al. 2011). Confocal imaging showed that in dark-grown seedlings, as expected, nuclear GFP-RGA was largely absent from hypocotyl cells, and was detected with increasing frequency through the apical hook region and the cotyledon (Vriezen, Achard et al. 2004; Josse, Gan et al. 2011). While ELF3 seems to play a prevalent role in mediating DELLAs destabilization in the cotyledons (as shown in Figure 40, this protein is not detected in the hypocotyl), COP1 seems to exert a regulatory role both in the hypocotyl and the cotyledons, as evidenced by the shorter hypocotyls of *cop1-6* seedlings and the tall hypocotyl and larger cotyledon size of *COP1*ox lines. Even more detailed studies are required, it is evident that ELF3 exerts an opposite control on RGA stability in the cotyledons (ELF3 destabilizes these repressors) than in the hypocotyl, while COP1 exerts a similar RGA-destabilizing effect in both organs. The *pRGA*::GFP-RGA lines in these different genetic backgrounds will be key to the identification of the molecular mechanisms underlying such complexity in regulation, co-IP experiments of the GFP-RGA protein thus helping to clarify if COP1 directly interacts with the DELLAs or requires of the ELF3 protein for interaction, and under which conditions such interaction takes place.

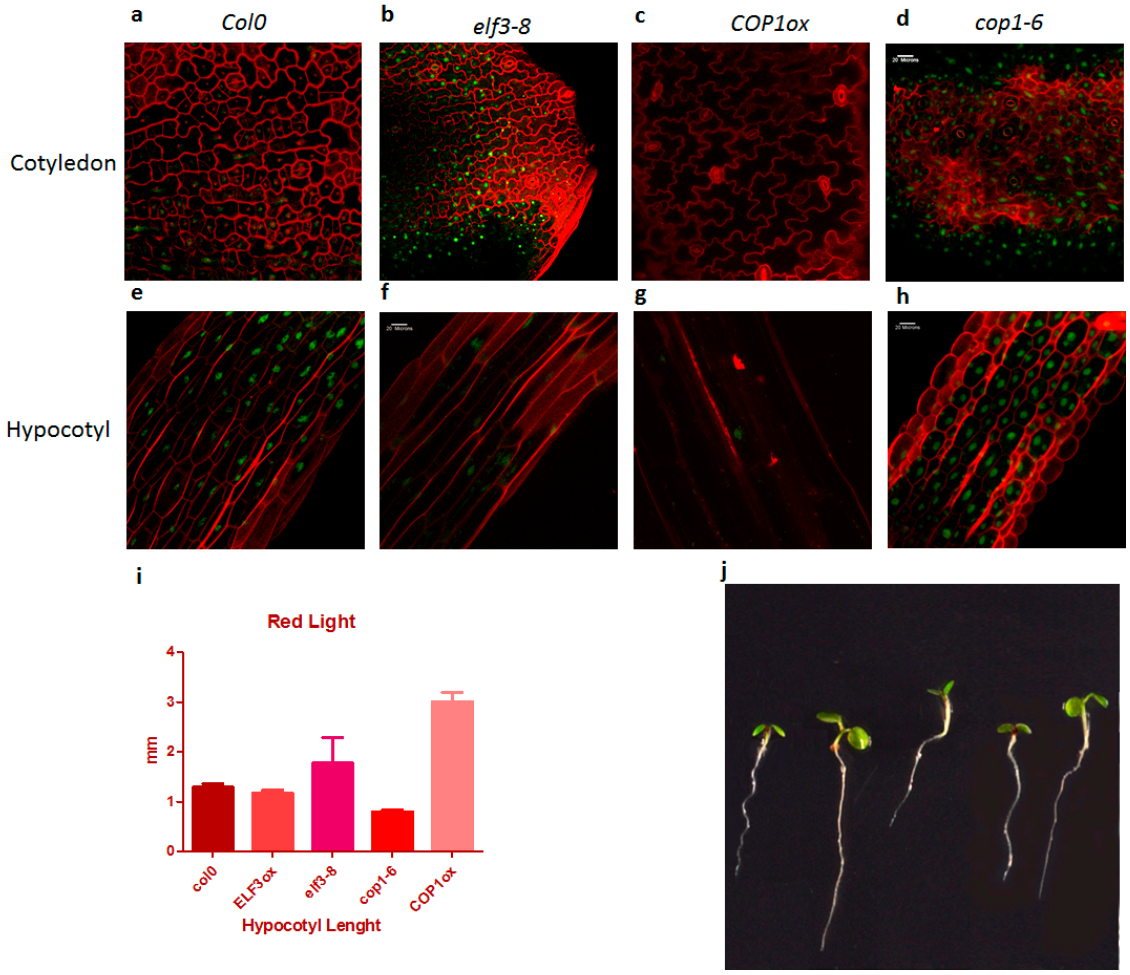

Figure 44. RGA protein levels are regulated by ELF3 and COP1 in an organ-specific manner in red light. GFP-RGA activity is detected in the cotyledons in a) *Col-0* under continuous red light and is increased in both b) *elf3-8* and d) *cop1-6* mutants but reduced in c) *COP1ox* lines. GFP-RGA activity is relatively high in the hypocotyls of e) *Col-0* seedlings, consistent with light growth inhibition of these organs. Levels of this fusion protein are further increased in the h) *cop1-6* mutant while they are reduced in both f) *elf3-8* and g) *COP1ox* lines. Remarkably, GFP-RGA activity correlates with the i) hypocotyl lengths of these different genotypes. j) Picture of the representative phenotypes of these plants, grown under red light.

PHYB contributes to establish a different organ-specific pattern of accumulation of the RGA protein.

During the SAS response, we observed that reduced PHYB signaling leads to RGA destabilization, but that destabilization of these repressors requires of ELF3 protein activity (Figure 33). Hence, we hypothesized that PHYB might stabilize the DELLAs by binding ELF3 and thus competing for ELF3-COP1 complex formation, that drives destabilization of these repressors. To test this hypothesis, we crossed the

pRGA::GFP-RGA lines with *phyB* and *PHYB*ox plants, in order to assess RGA protein stability in these genetic backgrounds. Actually, stabilization of the RGA repressor in the light is PHYB dependent, although it has been suggested that changes in RGA stability respond to PHYB-mediated regulation of GA levels (Achard, Liao et al. 2007). By confocal microscopy analyses of the GFP-RGA protein, we could confirm that levels of this protein in red light in the *phyB* mutant are reduced in and elevated in *PHYB*ox lines the hypocotyl, while the opposite was found in the cotyledon (Figure 45b-e). Moreover, upon a shift from red to far-red light, the protein accumulates in *phyB* mutant seedlings, but is destabilized in *PHYB*ox lines (Figure 45g). This indicates that PHYB plays an important role in modulating RGA levels, most likely by counteracting the negative effects of COP1. *phyb* and *PHYBox* plants grown under continuous red light show opposite phenotypes concerning the hypocotyl and cotyledon sizes of these plants (Figure 45a,f).

Noteworthy, levels of GFP activity of the GFP-RGA fusion are fully consistent with the phenotypes of these plants (Figure 45b-e), with reduced cotyledon size in the *phyb* mutant and short hypocotyls of *PHYB*ox lines being correlated with increased GFP activity in these organs (Figure 45c, d), while tall *phyB* hypocotyls and larger *PHYB*ox cotyledons do correlate with reduced GFP-RGA levels (Figure 45b, e). Therefore, we can conclude that RGA levels control the organ size of these plants, with PHYB playing an opposite regulatory role in the cotyledons (destabilizes the RGA repressor) compared to the hypocotyl (stabilizes this repressor) as observed for the ELF3 protein. *ELF3*ox and *PHYB*ox plants actually display a related phenotype, hence suggesting that these two proteins act in concert in the cotyledons, to counteract COP1 activity. DELLAs were actually reported to modulate cotyledon size, the *global* mutant showing taller hypocotyls but also larger cotyledons (Penfield, Gilday et al. 2006). PHYB is also known to modulate cotyledon size (Penfield, Gilday et al. 2006; Hou, Lee et al. 2010), with the smaller cotyledons of the *phyB* mutants being associated to the accumulation of RGA in the dark (Josse, Gan et al. 2011). Also, red light exposure led to a significant depletion in the pool of cotyledon-located GFP-RGA, and it appears that phyB activity simultaneously boosts RGA levels in the hypocotyl to restrict growth and reduces RGA levels in the cotyledons, allowing their expansion (Josse, Gan et al. 2011). We observed that levels of ELF3 are reduced in the *phyB* mutant and that RGA accumulates in the cotyledons of these plants. As reported, phyB controls hypocotyl-located RGA abundance, at least in part, by regulating the levels of bioactive GA for *phyB* mutant seedlings (Achard, Liao et al. 2007), we found that GA signaling and biosynthetic genes are also misexpressed in the *elf3-8* mutant. Together, these

observations suggests that PHYB plays an important role in modulating ELF3 activity and that this protein in turn plays a key role in controlling RGA stability, stabilization of this repressor hence leading to misregulated expression of GA biosynthetic and signaling genes in the *phyB* and *elf3-8* plants. However, since ELF3 binds the PHYB, COP1 and RGA proteins, and is not totally clear if COP1 is able to directly interact with the RGA repressor, further research will be required to assess functional relationship of these proteins and to establish if they exert a different regulatory function in the hypocotyl compared to the cotyledons.

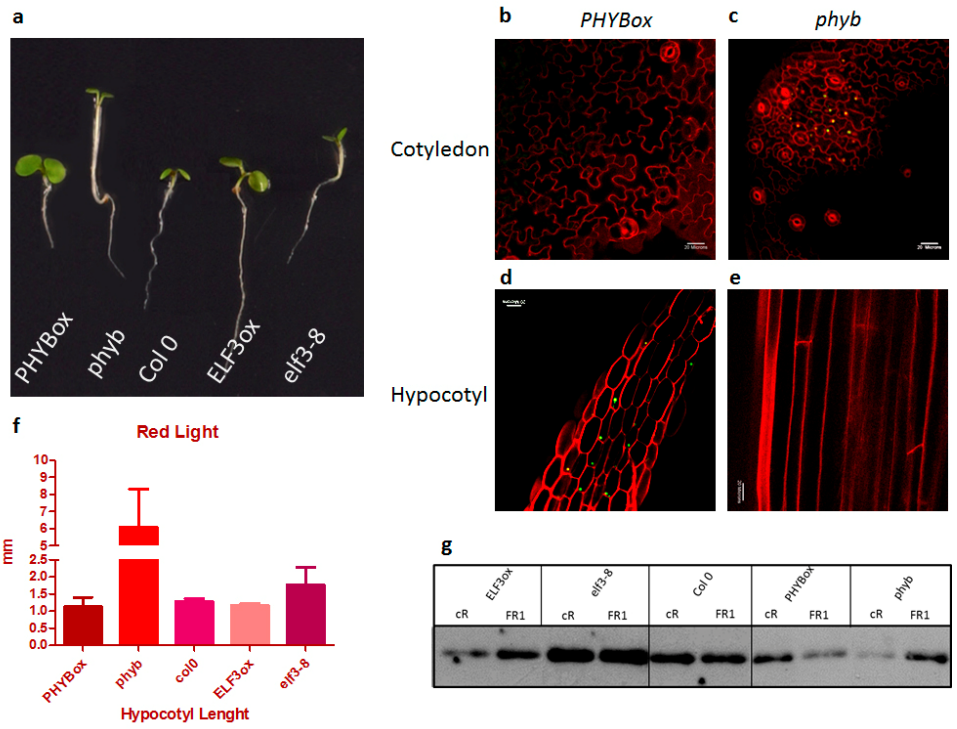

Figure 45. GFP-RGA levels are differentially distributed in *phyB* and *PHYBox* plants. a) Representative plants of the different phenotypes grown under red light. Fluorescence of the GFP-RGA protein is not detected in the cotyledons of b) *PHYBox* lines but in the b) *phyb* mutant accumulates in these organs. An opposite pattern of GFP-RGA accumulation is observed in the hypocotyls d) and f). GFP-RGA levels correlate with the f) hypocotyl lengths and cotyledon size of these plants. g) RGA levels in response to a shift from red to far-red light show also opposite profiles in these plants. Prolonged far-red inhibits COP1 activity,

Discussion and perspectives

The identification of ELF3 as a GAI interacting partner in yeast two hybrid assays, suggested a role of this clock related protein in the regulation of DELLA's function. These repressors were shown to accumulate during the day, but to be destabilized in the night suggesting that nuclear levels of these proteins are modulated by a diurnal or

light-dependent component. Accumulation of DELLAs was postulated to respond to diurnal changes in endogenous GA levels, but studies that would demonstrate that this is the main mechanism modulating stability of these repressors were lacking. In this work we show that the first leucine heptad repeat (LHI) of DELLAs mediates interaction with the central M-ELF3 domain of the ELF3 protein. This domain is highly conserved in all higher and lower plant ELF3 orthologues (Liu, Covington et al. 2001) and we show that a related domain is also present in other proteins. As M-ELF3, this domain mediates also interaction with the DELLAs, thus representing novel DELLA interacting motif. The central domain of ELF3 has been reported to mediate interaction with ELF4 (Nusinow, Helfer et al. 2011; Herrero, Kolmos et al. 2012), ELF3 then bringing together ELF4 and LUX to form the evening complex (EC). This domain also mediates ELF3 interaction with the flowering time protein GIGANTEA (GI), bridging COP1 and GI interaction (Yu, Rubio et al. 2008). Either way both findings indicate that ELF3 acts as a scaffold protein, hence bringing together the partner proteins bound to its different protein-protein interaction domains. ELF3 actually recruits the ELF4 and LUX/NOX proteins to form the ternary ELF4-ELF3-LUX complex, or evening complex (EC), that controls the rhythmic expression of several evening repressed genes, including the *PIF4* and *PIF5* factors, and the clock function *PRR9* gene (Nusinow, Helfer et al. 2011; Chow, Helfer et al. 2012; Herrero, Kolmos et al. 2012). ELF3 also serves as substrate adaptor between the flowering protein GI and the E3 ligase COP1, hence directing ubiquitination and subsequent proteasomal degradation of the GI protein, to control floral transition in short days (Yu, Rubio et al. 2008). Interaction of RGA to the ELF3 adaptor protein, on the other hand, is mediated by the first leucine heptad repeat (LHI). Interestingly, this domain is also implicated in interaction with the PIF3 and PIF4 transcription factors and in interaction with JAZ1, and thus is essential for the growth restraint activity of DELLAs (de Lucas, Daviere et al. 2008; Feng, Martinez et al. 2008; Gallego-Bartolome, Minguet et al. 2010; Hou, Lee et al. 2010). Therefore, this raised the possibility that 1) ELF3-RGA interaction blocks RGA repressor activity or 2) ELF3 bridges RGA interaction with other ELF3 partners to modulate this repressor stability. The fact that gene expression data relative to the ELF3 lines were restricted to unique microarray experiment on the weak *elf3-7* mutant (Kim, Hicks et al. 2005), together with the complexity of these analyses, due to the time of the day dependent function of ELF3, made difficult to judge on any of these regulatory models. Actually, the tall phenotype of *elf3-8* mutants suggested a function of ELF3 in growth repression, which was in apparent contradiction with a role of ELF3 in inhibiting RGA activity. Results reported in Chapter I, on the other hand, showed that PIF4 binds ELF3 and RGA via

the same domain, hence pointing to a competitive function of these proteins for PIF4 interaction. At the same time, ELF3 was found to modulate PIF4 transcriptional activity independently of RGA, which made further unclear the role of ELF3 and RGA interaction. Surprisingly, by analyzing RGA levels in the ELF3 genetic contexts we could establish that this repressor accumulates to higher levels in *elf3-8* mutant seedlings. Accumulation of the RGA protein does not depend on *de-novo* protein synthesis, thus evidencing a strong stabilization of this repressor in this mutant background. Noteworthy, GA application still directed degradation of this protein, indicating that increased RGA levels do not result from an impaired GA response or repression of the F-box *SLY1*, but reflect a direct role of ELF3-RGA interaction in RGA stability. However, more detailed GA dose-response studies are being conducted, to examine whether sensitivity to these hormones is still affected in the mutant. Analyses of GA signaling and GA biosynthetic genes, on the other hand, evidenced that stabilization of the RGA repressor in the *elf3-8* seedlings leads to a misregulated pattern of expression of these genes. Hence, altered GA homeostasis in these plants indicates that this repressor is biologically active. Intriguingly, we observed that the catabolic *GA2ox1* gene is also strongly up-regulated in these plants. Elevated levels of expression of this gene cannot be explained in terms of feed-back regulation, since *GA2ox1* is up-regulated in response to DELLA destabilization (Rieu, Ruiz-Rivero et al. 2008). Therefore, it will be interesting to further analyze what is the cause of such strong activation. Together, these findings indicate that ELF3 is required for diurnal regulation of RGA levels, in order to maintain GA homeostasis and GA signaling rhythmicity. Diurnal oscillation of GA signaling and metabolic genes has actually been reported to play an important role in rhythmic growth (Zhao, Yu et al. 2007; Arana, Marin-de la Rosa et al. 2011), with our findings demonstrating that diurnal changes in RGA levels, due to RGA-ELF3 interaction, play a crucial role in shaping circadian regulation of these genes.

Strikingly, *elf3-8* seedlings have a tall phenotype although they accumulate higher levels of the RGA repressor. Noteworthy, these plants also express the *GID1a* gene to higher levels and therefore it is possible that this receptor, as reported (Ariizumi, Murase et al. 2008; Ueguchi-Tanaka, Hirano et al. 2008), blocks RGA activity via a degradation independent mechanism. Although we could not exclude that ELF3 would be required for RGA repressive function, the finding that it acts as a scaffold protein suggested that ELF3 might modulate RGA activity by mediating interaction of this repressor with an additional negative regulatory partner. ELF3 actually binds the E3 ligase COP1 and thus can bridge interaction of the COP1 and RGA proteins, to

promote destabilization of this repressor at night. Levels of the RGA repressor are actually increased in *cop1-4* seedlings after transition from light to dark (Figure 32) and in EOD FR-treatments, increased RGA levels were observed in both *elf3-8* and *cop1-4* mutants (Figure 33). Actually, RGA, ELF3 and COP1 are likely to coexist in a ternary complex, since we observed that the nuclear localization pattern of ELF3 is changed after co-expression of both RGA and COP1 proteins, but not when co-expressed with either of these proteins alone. Similar results were observed for the RGA and GAI proteins (Figures 41 and 42), with these repressors being localized in nuclear bodies after co-expression with the ELF3 and COP1 proteins.

To test how changes in the levels of these proteins affect RGA accumulation, we crossed *Col-0 pRGA*::GFP-RGA lines with the *elf3-8*, *cop1-6*, and *phyB* mutants, and over-expression lines. As expected, increased levels of GFP activity were observed in *cop1-6* and *elf3-8* seedlings. However, in *elf3-8* plants GFP fluorescence was mainly restricted to the cotyledons, hence pointing to a relevant role of the ELF3 protein in modulating RGA levels in these organs. Interestingly, this accumulation pattern correlates with the tall hypocotyl and smaller cotyledons of these plants, hence explaining their contradictory phenotype. RGA protein levels were found to be increased in *cop1-6* seedlings and reduced in *COP1*ox plants, but in this case a similar distribution of the protein is observed in the cotyledons compared to the hypocotyls. These lines are currently being used for more detailed studies to correlate RGA accumulation with hypocotyl and cotyledon growth and co-IP studies to assess whether interaction of the RGA and COP1 proteins in the cotyledons requires of the scaffold function of ELF3. These lines will also be used in ChIP studies to analyze RGA-regulated gene expression (Zentella, Zhang et al. 2007) and its time dependency.

ELF3 was also shown to interact with PHYB (Liu, Covington et al. 2001), although molecular function of this interaction is not well understood. Light-dependent accumulation of RGA has been associated to an increase in GA levels in the night (Achard, Liao et al. 2007) and elevated GA levels were also reported for *phyB* mutants (Josse, Gan et al. 2011). Thus, we crossed the *pRGA*::GFP-RGA plants with *phyB* mutant and *PHYB*ox seedlings, to further analyze levels of the RGA repressor in these genetic backgrounds. Interestingly, RGA levels were found to be reduced in the *phyB* mutant, but predominantly localized in the cotyledons as observed for the *elf3-8* mutant. An opposite pattern of RGA accumulation was observed in *PHYB*ox lines, with higher levels of GFP fluorescence found in the hypocotyls compared to the cotyledons, hence correlating with the shorter hypocotyls and larger size cotyledons of these plants. Interestingly, the pattern of RGA distribution and phenotype of these plants

coincides with those of ELF3 lines, suggesting a concerted action of these two proteins in the cotyledons. Since both PHYB and COP1 interact with the same N-terminal domain of the ELF3 protein, we suggest that PHYB in concert with ELF3 destabilizes the RGA repressor in the cotyledons, by disrupting COP1 and RGA interaction mediated by ELF3. PHYB in its Pfr form is translocated into nucleus and competes with COP1 for ELF3 interaction, leading to a stabilization of both ELF3 and RGA proteins. In the night, PHYB is reversed to its inactive Pr form and excluded from the nucleus, coinciding with high COP1 activity. Formation of the COP1-ELF3-RGA complex hence contributing to maintain RGA levels low during the night.

Conclusions.

1. ELF3 and PIF4 control hypocotyl growth via a common regulatory pathway. This clock function-related protein modulates PIF4 and PIF5 expression by repressing transcription of these genes at dusk.

2. ELF3 binds the HLH domain of the PIF4 factor, with interaction of these proteins confirmed by BiFC and co-IP studies in *N. benthamiana* leaves. This domain is highly conserved in all members of the PIF family, the PIF1, HFR1 and SPT factors being also found to interact with ELF3.

3. Interaction with the HLH domain inhibits PIF4 DNA recognition ability, which identifies ELF3 as a novel negative regulator of PIF4 transcriptional activity.

4. ELF3 protein levels are regulated by PIFs, this clock-related protein accumulating to higher levels in the *pifQ* mutant and destabilized in *PIF4*ox lines. Levels of the ELF3 protein in these plants correlate with levels of accumulation of PHYB, suggesting a role of this photoreceptor in ELF3 stabilization

5. Antagonistic function of the ELF3 and PIF4 proteins suppresses the elongated PIF4ox plants. In turn, PIF4 over-expression rescued the LD late flowering phenotype of *ELF3*ox plants.

6. ELF3 interacts with all five *Arabidopsis* DELLA proteins via the conserved M-ELF3 central domain. Related domains are also identified in other proteins and found to interact with the DELLAs, hence defining this region as a novel DELLA interacting motif.

7. ELF3 negatively modulates RGA protein levels independently of *de-novo* protein synthesis. In *elf3-8* seedlings RGA accumulates to high levels independent of light conditions.

8. Diurnal changes in RGA levels play a crucial role in shaping circadian

regulation of GA signaling and GA metabolism genes, expression of these genes being misregulated in *elf3-8* mutant seedlings.

9. ELF3 plays a determinant role in modulating organ distribution of RGA levels by restricting abundance of this repressor in the cotyledons. RGA accumulates to high levels in elf3-8 cotyledons but not in the hypocotyls, hence correlating with the tall hypocotyl and smaller cotyledon size of these plants.

10. COP1 interacts with RGA and is required for its destabilization after light to dark transition and in EOD FR-treatments. Actually, RGA, ELF3 and COP1 are likely to coexist in a ternary complex, since the nuclear localization pattern of the ELF3 protein is modified after co-expression of both RGA and COP1 proteins.

11. Dark grown *cop1-4* seedlings are unable to accumulate RGA after the switch to light. In seedlings grown in the light, RGA accumulates to higher levels in the hypocotyl and cotyledons of *cop1-6* plants and destabilized in *COP1*ox lines. Levels of this repressor correlate with the shorter hypocotyls of *cop1-6* seedlings and the tall hypocotyl and larger cotyledon size of *COP1*ox lines.

12. In the *phyB* mutant RGA accumulates in the cotyledons, which are reduced, in opposite, *PHYB*ox seedlings accumulate high levels of this repressor in the hypocotyls and reduced levels in the cotyledons, hence correlating with the shorter hypocotyls and larger size cotyledons of these plants. This pattern of RGA distribution coincides with that of ELF3 seedlings, suggesting a concerted action of these two proteins in the cotyledons.

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APPENDIX I.

Resumen.

La elongación del hipocótilo en semillas de Arabidopsis sigue un patrón diurno caracterizado por un rápido crecimiento al final de la noche. Los PIFs (Phytochrome Interacting Factors) tienen un papel central en la elongación de hipocótilo integrando señales hormonales, luz y reloj circadiano (Leivar and Quail 2011). Estos factores de transcripción se unen a las cajas-G (CACGTG) y cajas-E (CANNTG) para activar la expresión de diversos genes relacionados con auxinas y con otros que codifican enzimas que remodelan la pared celular (Castillon, Shen et al. 2007). La expresión de estos genes contribuye a la pérdida de rigidez de la pared celular, lo que constituye un paso esencial para la elongación celular. La actividad transcripcional de los PIFs está muy regulada, previniendo así un crecimiento excesivo de la planta. La luz es un elemento determinante en la modulación de estos factores. La forma activa del fotorreceptor PHYB interacciona de forma directa con los PIFs, induciendo su degradación (Castillon, Shen et al. 2007) o bien impidiendo una unión eficiente de estos al DNA (Park et al. 2012) Los PIFs están además regulados por la ruta hormonal de las giberelinas, ya que se ha mostrado que las proteínas DELLA forman un complejo represor que secuestra e impide la unión de los PIFs al ADN, inhibiendo por consecuencia su actividad transcripcional (de Lucas, Daviere et al. 2008). Al mismo tiempo, las DELLA se estabilizan al reducirse los niveles de giberelinas (Achard et al. 2007). Hasta el momento, no se ha determinado el mecanismo mediante el cual se coordinan la luz y las giberelinas en condiciones fotoperiódicas para restringir el crecimiento al final de la noche.

En un cribado de doble hibrido en levaduras realizado en el laboratorio, se encontró que la DELLA GAI interacciona con la proteína del reloj ELF3. Esta proteína, única de plantas, es un componente endógeno del reloj y también determina la sensibilidad de la respuesta de la planta a las señales del ambiente. El reloj circadiano mantiene una estrecha relación con las rutas de señalización que regulan la elongación celular y determinan el patrón rítmico de crecimiento de las plantas. Los mecanismos moleculares que regulan el crecimiento rítmico se desconocían en el momento en que comenzó este trabajo (Nozue, Covington et al. 2007; Niwa, Yamashino et al. 2009). La interacción ELF3-DELLA apunta como un mecanismo plausible para la regulación del crecimiento rítmico conectando el oscilador central con el crecimiento. Los fenotipos

alargados del mutante de ELF3 (*elf3-8*), sugieren una función represora del crecimiento, de esta forma, ELF3 podría contribuir a la estabilización de las DELLAs y a regular negativamente a los PIFs.

En este trabajo, hemos encontrado que el mutante *pif4pif5* suprime el fenotipo alargado de *elf3-8*, estableciendo así su relación funcional. Por otro lado, encontramos que la sobreexpresión de ELF3 (*ELF3ox*) suprime el fenotipo alargado de la sobreexpresión de PIF4 (*PIF4ox*), lo que sugiere una regulación negativa de ELF3 hacia PIF4. Posteriormente, se encontró que el ritmo diurno de la expresión de PIF4 esta desregulado por la noche en *elf3-8*. El crecimiento rítmico del hipocótilo está restringido al final de la noche (Nozue, Covington et al. 2007). La rápida activación de los genes diana de PIF4 y el crecimiento arrítmico mostrado en los mutantes elf3 se explicaría por los altos niveles de PIF4 durante la noche. Estos resultados coinciden con los publicados durante el desarrollo de este trabajo (Nusinow, Helfer et al. 2011), donde se establece que ELF3 recluta a ELF4 y el factor de transcripción LUX/NOX para formar el complejo represor llamado “Evening Complex” (EC). Este complejo se une a los promotores de *PIF4/PIF5* por la tarde modulando de esta manera, su expresión rítmica. Adicionalmente, hemos encontrado mediante ensayos de doble híbrido en levaduras, la interacción entre ELF3 y PIF4. Esta interacción está mediada por el dominio C-terminal de ELF3 y el bHLH de PIF4. Consistente con la unión de ELF3 al dominio de unión al DNA de PIF4, hemos establecido que ELF3 desempeña un papel en la inhibición de la actividad transcripcional de PIF4 durante el día. De esta forma, contribuye a restringir la ventana temporal del crecimiento y redefine el papel de ELF3 no solo como una proteína adaptadora si no como un represor de PIF4. Por otro lado, los niveles de la proteína ELF3 se encuentran alterados tanto en la sobre-expresión de PIF4 así como en el cuádruple mutante *pifQ*. Dado que PIFs no alteran la expresión génica de ELF3, este hecho sugiere un nivel adicional de complejidad proteína-proteína entre estos dos factores, posiblemente dependiente de PHYB. Esto abre la posibilidad de que los PIFs proporcionen información al reloj circadiano ajustándolo mediante la modulación de los niveles de ELF3. Las plantas adultas de la línea que sobre-expresa tanto ELF3 como PIF4 muestran un fenotipo intermedio correspondiente a estos dos genes. El incremento en el vigor y longevidad de estas plantas así como su floración temprana hacen de estos dos genes unos candidatos plausibles como herramientas biotecnológicas en plantas de interés comercial.

Por otro lado, se comprobó la interacción ELF3-GAI obtenida en el escrutinio de doble hibrido en levaduras con todas las proteínas DELLA. Se definió un nuevo dominio

conservado con afinidad a las proteínas DELLA. Ya que ELF3 se ha descrito como adaptador con COP1 (Yu, Rubio et al. 2008), se midieron los niveles de la proteína RGA en mutantes cop1 y elf3. Observamos que los niveles de la proteína RGA están alterados en los dos fondos genéticos y, notablemente, presentan el mismo patrón en el cambio de luz a oscuridad. Sin embargo, en plantas etioladas se requiere COP1 para la estabilización de RGA. Esto sugiere que puede existir una co-regulación o ser de manera independiente según el programa de desarrollo. Hemos demostrado que la expresión diurna de los genes de síntesis y señalización de giberelinas (GA) está incrementada en *elf3-8*, debido a su vez a los niveles alterados de la proteína RGA. Este incremento es específico de tejido y explica el fenotipo de cotiledones reducidos de elf3-*8*. De igual forma, la interacción de ELF3 con PHYB (Liu, Covington et al. 2001) podría estar regulando los niveles de RGA, pues estos están alterados tanto en el mutante como en la sobre-expresión de PHYB, y la proteína RGA fusionada a la GFP muestra un efecto dependiente de tejido que está relacionado con el tamaño de los cotiledones que muestran estas plantas. De esta manera, proponemos que ELF3 es necesario para aliviar la represión impuesta por RGA y posiblemente por todas las DELLAsal crecimiento de los cotiledones. Para esclarecer este mecanismo de regulación, se estudió la localización nuclear de GAI y RGA, donde se encontró que es dependiente de la co-expresión de COP1 y ELF3. Asimismo, se encontró que RGA interacciona con COP1 y se estudiaron las condiciones en las que pudieran formar un mecanismo independiente o de co-regulación como sugieren nuestros estudios de los niveles de proteína.

Gracias a resultados planteamos un nuevo mecanismo por el cual la acción de los represores DELLAs está restringida según el tipo de tejido y proponemos una regulación por luz de manera independiente de las giberelinas hasta ahora no descrita.

Objetivos:

1. Análisis de la interacción entre ELF3-RGA y ELF3-PIF4.
2. Establecer las implicaciones biológicas de la interacción ELF3-PIF4 analizando la actividad transcripcional y la estabilidad de PIF4 en las líneas *ELF3ox* y en el mutante *elf3-8*.
3. Generar líneas dobles *PIF4*ox*ELF3*ox y *PIF4*ox*elf3*, para analizar la expresión de los genes regulados y la relación con la longitud del hipocótilo.
4. Analizar el patrón diurno del represor RGA en la línea *ELF3ox* y el mutante *elf3-8*.

5. Estudio del patrón diurno de genes relacionados con GA en las líneas ELF3. Establecer un modelo de acción de estos reguladores que contribuya a entender el crecimiento rítmico.
6. Establecimiento de un modelo de acción de estos reguladores que contribuya a entender el crecimiento rítmico.

Conclusiones.

1. ELF3 y PIF4 controlan el crecimiento del hipocótilo mediante una ruta de señalización común. Esta proteína de reloj modula la expresión de PIF4 y PIF5 reprimiendo la transcripción de estos genes por la tarde.
2. ELF3 se une al dominio HLH de PIF4, cuya interacción fue confirmada por BiFC y co-IP en hojas de *Nicotiana benthamiana*. El dominio HLH está altamente conservado en todos los miembros de la familia de los PIFs y los homólogos de PIF4 PIF1, HFR1 y SPT también interaccionan con ELF3.
3. La interacción con el dominio HLH inhibe la habilidad de PIF4 para reconocer el DNA, lo que identifica a ELF3 como un nuevo factor que regula negativamente la activación transcripicional de PIF4.
4. Los niveles de proteína de ELF3 están regulados por los PIFs, esta proteína de reloj se acumula en altos niveles en el mutante *pifQ* y se desestabiliza en PIF4OX. Los niveles de proteína de ELF3 en estas plantas se correlacionan con los niveles de acumulación de PHYB, sugiriendo un papel de este fotorreceptor en la estabilización de ELF3.
5. La función antagonista de ELF3 y PIF4 suprime el fenotipo alargado de las plantas PIF4OX. Por otro lado, la sobreexpresión de PIF4 rescata el fenotipo de floración tardía en días largos de ELF3OX.
6. ELF3 interacciona con las cinco proteínas DELLA de *Arabidopsis* a través el dominio central conservado M-ELF3. Este dominio se identificó en otras proteínas que también interaccionaban con las DELLA, definiendo así esta región como un nuevo dominio de interacción para estas proteínas.
7. ELF3 modula negativamente los niveles de la proteína RGA independientemente de las síntesis de-*novo* de proteínas. Las plántulas *elf3-8* acumulan altos niveles de RGA independientemente de cualquier condición.
8. Los cambios diurnos en los niveles de RGA juegan un papel crucial en la regulación circadiana de los genes de señalización y metabolismo de GA. La expresión de estos genes está desregulada en plantas de *elf3-8.*

9. ELF3 juega un papel determinante modulando la distribución tisular de los niveles de RGA restringiendo la abundancia de este represor a los cotiledones. RGA se acumula en los cotiledones de *elf3-8* pero no en los hipocótilos, correlacionándose con el hipocótilo alargado y reducidos cotiledones en estas plantas.
10. Las plántulas *cop1-4* en condiciones de oscuridad no son capaces de acumular RGA después del cambio a luz. Mientras que plántulas crecidas en luz acumulan altos niveles de RGA en el hipocótilo y en los cotiledones de *cop1-6*, se encuentran desestabilizados en COP1OX. Los niveles de este represor se correlacionan de ese modo con los hypocótilos reducidos de *cop1-6* y los hipocótilo alargados y cotiledones expandidos en COP1OX.
11. En el mutante *phyB,* RGA se acumula en los cotiledones al contrario de PHYBOX que acumula altos niveles de este represor en los hypocótilos y reducidos en los cotiledones. Este patrón de distribución coincide con el fenotipo de hypocótilos reducidos y gran tamaño de los cotiledones de PHYBOX, así como unos cotiledones reducidos del mutante *phyb.* El patrón de este último coincide con el de las plántulas *elf3-8*, sugiriendo una acción conjunta de estas dos proteínas en los cotiledones.